20030128336

DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Illinois at Urbana-Champaign	7a. NAME OF MONITORING ORGANIZATION
6b. OFFICE SYMBOL (If applicable)	
6c. ADDRESS (City, State, and ZIP Code): Urbana, Illinois 61801	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-85-C-5241
8b. OFFICE SYMBOL (If applicable)	
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102A	3M1 61102BS12	AA	006

11. TITLE (Include Security Classification)

Pathophysiology and Toxicokinetic Studies of Blue-Green Algae Intoxication in the Swine Model

12. PERSONAL AUTHOR(S) B.R. Beasley, W.M. Haschek-Hock, W.W. Carmichael, W.O. Cook, A.M. Dahlem, M.D. Pikes, F.D. Galey, S.B. Hooser, R.A. Lovell, and N.A. Mahmood

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 9/1/85 TO 8/31/86	1986 November 21	193

16. SUPPLEMENTARY NOTATION

Keywords:

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	16		Microcystis aeruginosa, microcystin, cyanoginosin, Nodularia spumigena, peptide hepatotoxin, Anabaena flos-aquae, anatoxin-a anatoxin-a(s), anticholinesterase, nicotinic agonist
06	20		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

NEUROTOXIN STUDIES. Neurotoxin production is being scaled up; purification methods are being compared. Mouse bioassay is used to identify toxic fractions. Stability studies with anatoxin-a(s) revealed toxin degradation, particularly in organic solvents; the process can be slowed by acidification. TLC has revealed previously unrecognized contaminants. We anticipate purification of toxin in its free base form, with normal phase HPLC; because of neglible UV absorption, new methods of detection are needed. Collaboration with Dr. Ken-Ichi Harada at Meijo University in Nagoya, Japan, will continue.

Inhibition by anatoxin-a(s) of eel and human erythrocyte acetylcholinesterases was time and concentration dependent and followed first order kinetics, indicative of reversible binding followed by irreversible inhibition. RBC cholinesterase was more sensitive. Inhibition of horse serum cholinesterase, however, was non-linear.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	301-663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

AD_____

PATHOPHYSIOLOGY AND TOXICOKINETIC STUDIES OF BLUE-GREEN ALGAE INTOXICATION IN THE SWINE MODEL

ANNUAL REPORT

NOVEMBER 21, 1986

Val R. Beasley
Wanda M. Haschek-Hock
Wayne W. Carmichael
William O. Cook
Andrew M. Dahlem
James D. Fikes
Francis D. Galey
Stephen B. Hooser
Randall A. Lovell
Nik A. Mahmood

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5241

University of Illinois at Urbana-Champaign
Urbana, Illinois 61801

TABLE OF CONTENTS

TABLE OF CONTENTS, CONTINUED

TABLE OF CONTENTS, CONTINUED

LIST OF TABLES

ANALYTICAL INVESTIGATIONS WITH CYANOBACTERIAL TOXINS

PRELIMINARY STUDIES WITH CYANOBACTERIAL NEUROTOXINS

PRELIMINARY STUDIES WITH ANATOXIN-A

BLUE-GREEN ALGAE (MICROCYSTIS AERUGINOSA) HEAPTOTOXICOSIS IN A HERD OF DAIRY COWS

EFFECTS ON MALE MICE OF ONE OR TWO INTRAPERITONEAL DOSES OF A HEPATOTOXIN PRODUCED BY MICROCYSTIS AERUGINOSA

OVERVIEW OF FIRST YEAR STUDIES IN GILTS FOLLOWING HEPATOTOXIN ADMINISTRATION

THE EFFECTS OF 7820 MICROCYSTIN, A CYCLIC HEPTAPEPTIDE TOXIN FROM MICROCYSTIS AERUGINOSA, IN THE RAT

LIST OF INVESTIGATORS

Name	Position/Speciclty	Responsibility
Val R. Beasley	Assistant Prof. of Toxicology Dept. of Vet. Biosciences	Project Director
Wanda M. Haschek-Hock	Associate Prof. of Pathology Dept. of Vet. Pathobiology	Co-Investigator, Pathology Studies
Wayne W. Carmichael	Associate Professor Dept. of Biol. Sciences Wright State University	Co-Investigator, Toxin Production
Lloyd Barr	Prof. of Bioeng, Physiology and Biophysics Dept. of Physiology	Consultant for Anatoxin-A Studies
William O. Cook	Research Associate, Toxicology Dept. of Vet. Biosciences	Anatoxin-a(s) Studies
Andrew M. Dahlem	Grad. Research Assistant, Toxicology Dept. of Vet. Biosciences	Analytical Methods and Toxin Purification
Joni K. Dye	Veterinary Technician Dept. of Vet. Biosciences	Pathophysiology Studies
James D. Fikes	Research Associate, Toxicology Dept. of Vet. Biosciences	Anatoxin-A Studies
Francis D. Galey	Research Associate, Toxicology Dept. of Vet. Biosciences	Clinical Hepato- toxin Studies in Cattle
Kenneth R. Holmes	Associated Professor of Physiology/Neurobiology Dept. ov Vet. Biosciences	Consultant, Swine Hepatotoxin Studies
Stephen B. Hooser	Research Associate, Pathology Dept. of Pathobiology	Pathology Studies
Randall A. Lovell	Research Associate, Toxicology Dept. of Vet. Biosciences	Swine Hepatotoxin Studies
Nik A. Mahmood	Postdoctoral Research Assoc. Dept. of Biological Sciences Wright State University	Toxin Production and Purification
Alan J. Parker	Professor of Neurology/Small Animal Medicine Veterinary College of Medicine	Consultant, Anatoxin-a(s) Studies
William M Valentine	Postdoctoral Research Assoc., Toxicology Dept. of Vet. Biosciences	Pathophysiology Coordinator

PRELIMINARY STUDIES WITH ANATOXIN-A

EFFECTS ON MALE MICE OF ONE OR TWO INTRAPERITONEAL DOSES OF A HEAPTOTOXIN PRODUCED BY MICROCYSTIS AERUGINOSA

THE EFFECTS OF 7820 MICROCYSTIN, A CYCLIC HEPTAPEPTIDE TOXIN FROM MICROCYSTIS AERUGINOSA, IN THE RAT

LIST OF FIGURES

ANALYTICAL INVESTIGATIONS WITH CYANOBACTERIAL TOXINS

PRELIMINARY STUDIES WITH CYANOBACTERIAL NEUROTOXINS

STUDIES ON CYANOBACTERIA (BLUE-GREEN ALGAE) NEUROTOXINS

ANALYTICAL INVESTIGATIONS WITH CYANOBACTERIAL TOXINS

Andrew M. Dahlem

CONTENTS

Introductory analytical investigations with three toxins from blue-green algae were designed to provide preliminary information into toxin purification, stability and chemical properties. In addition to basic chemical investigations with the cyclic peptide hepatotoxins, studies were initiated to determine the structure toxicity relationships of several moieties contained in the peptide toxins.

I.A. CYCLIC PEPTIDE HEPATOTOXINS FROM BLUE-GREEN ALGAE

Andrew M. Dahlem, Ken-ichi Harada, Wayne W. Carmichael, Val R. Beasley

Introduction

Toxic blooms of cyanobacteria are a cosmopolitan characteristic feature of many nutrient-filled fresh and brackish waters. Two genera, Microcystis and Nodularia, which have produced naturally occurring peptide hepatotoxin containing blooms, are being studied, and their toxins have been isolated and purified. The principal species under investigation, Microcystis aeruginosa, has been frequently cited in animal poisoning incidents and the toxin of interest from this species is a low molecular weight (994 daltons), peptide hepatotoxin with a relatively high toxicity (LD_{50}:35-50 ug/kg IP mouse). The other hepatotoxin producing cyanophyte being studied is Nodularia spumigena. This algae was collected by the research group of M. H. G. Munro from a toxic bloom in Christchurch, New Zealand and the toxic principle is under structural investigation by the spectroscopy group of Dr. Kenneth Rinehart with Dr. Ken-Ichi Harada in the Department of Chemistry at the University of Illinois. It too is a low molecular weight toxin of high toxicity (LD_{50}:50-80 ug/kg IP mouse) and has many structural similarities to the toxin from Microcystis aeruginosa.

Isolation and purification methods for the cyclic peptide toxins as well as development of methods to assess the purity of toxin supplied are clearly essential prior to toxicology studies to assess such parameters as minimal lethal dose (MLD), no effect dose (NED), LD_{50}, and pathophysiologic functions. These algae toxins display very steep dose/effect curves between a dose which will not cause death, and potentially lethal doses. For these reasons the integrity of toxin must

be precisely known so that toxicology studies can be valid and repeatable.

Microcystin (Cyanoginosin-LR, toxin-LR, toxin BE-2) is a potent peptide hepatotoxin produced by the blue-green algae Microcystis aeruginosa. Several methods have been developed for the isolation and purification of this and closely related toxins (1-3) but all methods to date rely on purity evaluation based on HPLC with UV absorption. The extraction procedure developed in Dr. Carmichael's Laboratory has been used to produce large amounts of toxin of relatively high purity, however non-UV-absorbing impurities have been occasionally observed in material purified by this method. In order to develop dose/response data on this potent toxin, and to establish the purity of material to be tested, additional methods of separation and detection are required.

In order to standardize toxin material and to describe other compounds which occur as contaminants of toxin, a thin layer chromatography (TLC) method was developed. Initial evaluations of toxin material has revealed contaminants which were not detectable by high performance liquid chromatography (HPLC) with U.V. detection alone. The goal of this study was to develop a TLC method utilizing two detection reagents including iodine vapor followed by sulfuric acid to determine contaminants which are not detectable by U.V. absorbence alone. Subsequently, these alternate methods of toxin separation and detection in addition to the standard method of HPLC with UV detection were used to ensure the purity and integrity of toxin used in animal toxicity testing.

MATERIALS AND METHODS

Organisms

The microcystins from two different strains of Microcystis aeruginosa, extracted by two different methods, were evaluated. One of

the Microcystis strains was grown in the laboratory of coinvestigator Dr. Wayne Carmichael and was designated as strain 7820. The other Microcystis cell material was from a surface bloom collected from a farm pond in Monroe, Wisconsin and is designated as the Monroe strain. Cells from both strains of Microcystis were lyophilized prior to toxin extraction.

Toxin Extraction and Purification

The procedure used for toxin extraction of the 7820 strain (Fig. 1) was developed in the laboratory of coinvestigator Dr. Wayne Carmichael as a modification of the Siegelman method and is described by Krishnamurthy et al. (4).

The procedure used for toxin extraction of the Monroe strain is shown in Figure 2.

Thin Layer Chromatography

Microcystin which was extracted from algae cells and evaluated to be of greater than 90% purity by HPLC with U.V. detection was dried by lyophilization or under a nitrogen stream prior to purity analysis. Sufficient HPLC grade methanol was then used to dissolve toxin to give a final concentration of 5 ug/ul. Samples were applied to the high performance silica TLC plate with glass microcaps in amounts of 5, 10, and 25 ug and application was verified by observation under short wave U.V. prior to development (toxin absorbs short wave UV). The TLC plates were placed in pre-equilibrated TLC chambers and the solvent path was 13 cm.

TLC Mobile Phase

a) System 1 - Ethyl acetate-isopropyl alcohol-water (4:3:7). This system forms two phases and the top (organic) layer was used for TLC separation.

b) System 2 - Chloroform-methanol-water (65:35:10). This solvent system also forms two layers and in this case the lower (organic) layer is used for TLC separation.

Detection

The developed TLC plate was heated at 105°C for 5 minutes to evaporate remaining solvents. The plate is visualized under short wave U.V. light (since the toxin absorbs in this wave length range) and the U.V. absorbing eluted components of the "toxin" were marked with pencil on the silica surface. The plate was then placed in a chamber with iodine vapor and, after 5 minutes, the positions of the toxin and impurities were evaluated. The iodine was driven from the TLC plate by heating at 105°C for 5-10 minutes. The TLC plate was then sprayed with 30% H_2SO_4 in methanol and heated for 10 minutes at 105°C. The locations of toxin (r.f. 0.25) and impurities were determined by observation of the TLC plate under long wave U.V. light.

Purity Evaluation

The purity of the toxin was subjectively evaluated by observing the relative concentration of the toxin with respect to impurities at each of the concentrations applied to the TLC plate.

RESULTS AND DISCUSSION

The method eliminates costly and time consuming gel filtration steps (developed for purification of the Monroe strain) and reduces toxin extraction time by eliminating the time necessary to evaporate large volumes of water. It has been used to produce only small quantities of purified toxin but has the advantage of isolating the toxin in organic solvents which can be directly applied to silica gel TLC plates to test toxin purity.

U.V. detection is a valuable tool which can be used in evaluating the concentration and purity of compounds under investigation. U.V. detection

alone, however, does not detect some compounds which can occur as contaminants of purified toxin material and, for this reason, other methods of detection and analysis were developed. Purified toxin which appeared greater than 95% pure by HPLC with UV detection alone was shown to contain up to 20% contamination when examined by TLC methods.

The TLC method described offers a normal phase separation method and two spray reagents which serve as detectors in addition to the U.V. detection used in reverse phase toxin purification. This additional TLC method has proven valuable for the description of toxin material.

I.B. STABILITY EVALUATION OF MICROCYSTIN IN AQUEOUS SOLUTION

Andrew M. Dahlem, Val R. Beasley

Materials and Methods

Stability of purified toxin material was investigated concurrently with toxin purification methods. Purified toxin was dissolved in aqueous solution and periodically evalu ted to determine the fate of the toxin material. Toxin stability was assessed by comparing the HPLC chromatograms of a freshly prepared toxin standard solution with those of stored toxin solutions under the conditions of freeze/thaw, refrigerate/warm or constant room temperature storage.

Results

Toxin degradation under any examined condition does not appear to be a problem except under extreme conditions of repeated freeze/thaw or greater than 1 week at room temperature.

I.C. PRODUCTION OF RADIOLABELLED MICROCYSTIN TOXIN

Andrew M. Dahlem, Ken-Ichi Harada, Wayne W. Carmichael, Val R. Beasley

Introduction

Problems associated with the detection of toxin material with U.V. absorption can be minimized with the use of radiolabelled toxin in

analytical methods development. The radiolabel will subsequently facilitate pharmacokinetic studies assessing absorption, distribution, metabolism and excretion by providing an easily detectable isotopic label which should remain a part of a metabolized toxin. This label will provide a detectable marker even if the toxin becomes structurally altered or protein bound and thereby difficult to isolate and/or detect by UV absorption. Two possible methods of labelling the toxin have been preliminarily investigated by our research group; biosynthetically, involving C-14 label, and synthetically, with a tritium label.

Thus far, the production of biosynthetically labelled toxin has been proposed by providing C-14 labelled amino acids as raw materials for growing algae. These labelled precursor amino acids are likely to be incorporated into the toxin structure. To test this hypothesis, a culture of Microcystis aeruginosa strain 7820 was provided by Dr. Carmichael and it is being continuously grown in our laboratories. The identity of toxic material from this culture was established by spectroscopy methods involving Fast Atom Bombardment Mass Spectroscopy (FAB-MASS), Gas Chromatography (G.C) and mouse bioassay. At this point we are ready to begin addition of C-14 labelled precursors to try to create labelled materials.

Production of synthetically labelled toxin was proposed by utilizing the free alpha-carboxyl moieties present in glutamic acid and B-methyl aspartic acid to produce a substitution of ^{3}H for hydrogen in the alpha carboxyl. Botes (5) utilized a technique to prove isolinkage of these moieties in the peptide structure which involved a tritium labelling of C-terminal amino acids. This method was investigated by our research group to determine its feasibility for producing labelled toxin for toxicologic investigation.

MATERIALS AND METHODS

Isotopes

Tritiated water was purchased from New England Nuclear Research Products Company, Boston, MA, USA. The specific activity was 1 uCi/ul.

Solvents and Chemicals

All solvents were distilled in glass.

Isotopic Labelling

The peptide toxin (0.1 u mole) was placed in a small test tube and ^3H_2O (5 ul, 5 uCi) and pyridine (10 ul) were added. Acetic anhydride (10 ul) was then added to the same test tube and the mixture, in a parafilm-sealed tube, was kept at 0°C for 5 minutes. After this 5 minute period, the tube was moved to a room temperature (23°C) water bath for 15 minutes more. Another volume (20 ul), of pyridine was added, followed by acetic anhydride (20 ul) and the mixture was again immersed in an ice water bath at 0°C for 5 minutes followed by a room temperature water bath for an additional hour. Another tritiated water aliquot (5 ul) was then added and the mixture immersed in a room temperature water bath for 1 hour to decompose the excess acetic anhydride. The solution was then evaporated to dryness. Removal of exchangeable tritium was accomplished by addition of 10% acetic acid (100 ul) followed by evaporation. This addition of acetic acid followed by evaporation was then repeated 5 times.

RESULTS AND DISCUSSION

The toxin was successfully labelled synthetically by our research group using tritiated water and purified toxin as precursors. This experiment utilized the unusual isolinkage of glutamic acid and B-methyl aspartic acid which results in a free alpha-carboxyl in each moiety that would otherwise be involved in peptide bonding. This free alpha carboxyl allows tritium

incorporation by the method of Matsuo (6), which is customarily used to provide information about the C-terminal amino acid of noncyclic peptides. Figure 3 shows the synthesis pathway for tritium incorporation and assumes all reactants except the tritiated water are anhydrous so that as little extraneous water as possible is available which can interfere with toxin labelling.

I.D. STRUCTURE/TOXICITY RELATIONSHIP OF DEHYDROAMINO ACID MOIETY OF PEPTIDE

Andrew M. Dahlem, Ken-Ichi Harada, Carl A. Harvis, Stephen B. Hooser, Kenneth L. Rinehart, Val R. Beasley

Introduction

To begin to determine which factors are responsible for the high toxicity of algal peptide toxins, we have conducted numerous investigations into the structure/toxicity relationships of functional moieties of the hepatotoxins. The structure data on Nodularia toxin remains incomplete to date, but enough has been established by Dr. Ken-Ichi Harada and others, here at the University of Illinois, to draw parallels between this compound and microcystin. Both compounds contain the unusual twenty carbon Adda group, a dehydro amino acid, and stereospecific d- isomer amino acids. The Nodularia toxin has two fewer amino acid residues than the microcystin and yet has approximately the same toxicity.

The first moiety investigated in the peptide hepatotoxin was the dehydroamino acid. It has been shown that the bioactivity associated with other naturally occurring peptide molecules is directly related to the presence of dehydroamino acids. The antibiotics nisin and subtilin, produced by bacteria, contain alpha-beta unsaturated amino acids which are believed to be essential to their antibiotic activity (7). The toxic agents phomopsins A and B are cyclic hexapeptides produced by the fungus

Phomopsis leptostromiformis which contain didehydro amino acids and cause lupinosis in Australia. One working hypothesis for the biological action of cyclic peptide hepatotoxins containing dehydro amino acids suggests that the toxins may exert an effect on essential sulfhydryl groups by reacting across the double bond in their alpha, beta unsaturated amino acids. For these reasons, the role of the dehydroamino acid in the peptide toxin produced by Nodularia spumigena was investigated.

MATERIALS AND METHODS

Toxin

Purified Nodularia toxin was prepared from algae collected during a toxic bloom of Nodularia spumigens in Christchurch, New Zealand. Toxin was determined to be greater than 95% pure by HPLC and TLC evaluation before toxicity testing.

Chemicals

All solvents were distilled in glass. All chemicals were of analytical grade.

Toxin Derivative Formation

The dihydro derivative of Nodularia toxin was formed by hydrogenation of the dehydroamino acid contained in the cyclic peptide. Toxin material (100 mg) was dissolved in methanol (50 ul) and placed in a test tube. Excess sodium borohydride was added to the test tube slowly. The test tube then was placed at room temperature (23°C) for 24 hours and the solution continuously stirred. Acetic acid was added (200 ul) to lower the pH to 4, and eliminate the excess sodium borohydride and thereby stop the reaction. The reaction mixture was then evaporated to dryness.

TLC

The reaction product was spotted on high performance silica gel TLC plates. The elution solvent was chloroform-methanol-water (65:35:10). This mixture forms two phases and the lower phase (organic) was used. The toxin derivative was observed under short wave U.V. light and iodine vapor used as a detection reagent.

HPLC

HPLC separation of the dehydroamino acid from the reaction products was accomplished under reverse phase isocratic conditions with an Altech C-18 (250 mm x 4.6 mm) column and a mobile phase of 70:30 methanol- .05 percent trifluoracetic acid in water. Detection was by UV absorbence at 254 nm.

Confirmation of Structural Alterations

Mass spectral confirmation of the reaction product was accomplished with fast atom bombardment mass spectroscopy (FAB MASS) on a ZAB-SE 10 kV mass spectrometer. The product was imbedded in magic bullet matrix and parent ion of 827 was observed ($M + H^+ + 2$ Hydrogen).

Mouse Bioassay

The modified toxin then was tested for its toxicologic potential by IP injection in mice. In this study 37 adult female balb/c mice were randomly assigned to one of 11 test groups containing 3 mice or to a control group of 4 animals (Table 1). Animals were injected intraperitoneally with a saline vehicle, parent toxin, or dihydro-toxin dissolved in physiologic saline. Mice dosed with parent toxin received either 25, 50, 100, 200 or 300 ug/kg of toxin to serve as positive controls. Mice dosed with the vehicle (physiologic saline) alone were injected with volumes of .25, .50, .75, or 1.0 ml to serve as negative controls. Mice dosed with the dihydro-derivative of Nodularia toxin

received 100, 200, 300, 400, 500 or 1,000 ug/kg body weight of material in less than 1.0 ml of the vehicle.

Survival times of animals were recorded along with liver and kidney and whole body weight. Animals which survived 24 hours after toxin administration were killed by cervical dislocation. Negative control animals were killed at times within the study where numerous toxin produced deaths were occurring or after 24 hours when toxin-dosed survivors were killed. The thoracic and abdominal organs, and the brain were examined grossly. Lungs were inflated via intratracheal instillation of 10% neutral buffered formalin. Selected tissues including liver and kidney were fixed by immersion in 10% neutral buffered formalin. These tissues were then routinely processed, embedded in paraffin, sectioned at 4 to 6 um and stained with hematoxylin and eosin.

Results and Discussion

This sodium borohydride reaction selectively caused the addition of two hydrogen atoms across the double bond of the dehydroamino acid of the cyclic peptide toxin and resulted in a product identical to the starting material parent toxin except that the dehydroamino acid was now saturated forming the dihydro-derivative of the toxin. The identity of the dihydro-derivative was confirmed by FAB-MASS and found to be equal to the mass of the parent toxin plus two mass units (from the two hydrogen atoms added).

The dihydro-derivative of the Nodularia toxin was found to be four times less toxic than the parent toxin. Survivors of the dihydro Nodularia toxin showed liver lesions which were grossly and histologically very different from animals surviving administration of the parent toxin.

All mice given doses of unaltered Nodularia toxin equal to or greater than 100 ug/kg died. Mice given the dihydro-derivative of the Nodularia toxin did not die until the dose was 500 ug/kg or greater. Mice which died acutely following exposure in both groups had markedly enlarged, dark red livers at necropsy. Mice dosed with the dihydro-derivative toxin and which survived, had liver lesions unlike those found in the livers of mice dosed with unaltered Nodularia toxin. On gross examination, the outer portion of the livers of the survivors of the 400 ug/kg dihydrotoxin group were extremely pale while the inner portions of the livers were darker than normal. The livers of mice given 100 ug/kg of the dihydrotoxin were normal in color, but friable.

Microscopically, the hepatic lesions in all mice dying from either the parent or the dihydrotoxin were characterized by severe, extensive centrilobular, and midzonal disassociation, degeneration, necrosis, and cell loss involving all regions except for a rim of periportal hepatocytes 3 to 6 cells wide. Degenerating and necrotic hepatocytes were separated by large numbers of extravasated red blood cells. Some of the periportal hepatocytes appeared normal, while others showed varying signs of degeneration. In addition, the livers of mice given the dihydro-derivative of the Nodularia toxin had extensive subcapsular hepatocellular necrosis and hemorrhage in peripheral areas that grossly were pale.

Renal cortical tubules were mildly dilated and contained moderate amounts of eosinophilic to basophilic granular material. Many capillaries in the lung contained abundant, eosinophilic, finely granular to globular material. No effect of vehicle administration was observed in negative control animals.

From this experiment it appears that the double bond in the dehydroamino acid of Nodularia toxin plays a significant role in the toxic action of the Nodularia toxin. Saturation of the dehydroamino acid resulted in a shift of the mortality curve to the right as well as a change in the lesion observed. It is, however, important to note that while the toxicity obviously decreases after hydrogenation, the material still remains a potent toxin with a LD_{50} between 400-500 ug/kg.

I.E. FORMATION AND ASSESSMENT OF HEXAHYDRO NODULARIA TOXIN

The next structure/toxicity experiment was performed to assess the toxicologic significance of the ADDA group, already mentioned to be consistent in cyclic peptide cyanobacterial toxins.

Materials and Methods

Nodularia toxin was reduced with hydrogen gas and a palladium catalyst. This reaction causes the addition of six total hydrogens to the Nodularia toxin structure and saturates both the dehydroamino acid and the double bonds of the hydrophobic tail of the ADDA moiety (Figure 4). Mice were given the hexahydrotoxin at from 400 to 2,000 ug/kg; observed for 14 hours and killed by decapitation. Necropsy methods and tissue processing were described above.

Results

Intraperitoneal administration of this compound failed to produce mortality in mice at doses up to 2,000 ug/kg which is the highest concentration of material tested to date.

The mice dosed with the highest concentration of this compound had scattered, small, dark red areas distributed on the surface and throughout the hepatic parenchyma. Microscopic liver lesions, which were confined to the dark red areas, were characterized by severe hepatocyte disassociation, degeneration, and necrosis and severe hemorrhage similar

to that described for the unaltered and dihydro-derivative of Nodularia toxin.

Discussion

In addition to the double bond of the dehydroamino acid, the ADDA group must play a key role in toxic action of the Nodularia toxin. Saturation of reactive sites on the ADDA group decreases the toxicity of the Nodularia toxin and changes the lesion observed pathologically. Additional experiments are planned which will continue to assess structure-toxicity relationships in both the Nodularia toxin and microcystin.

II. NEUROTOXINS FROM BLUE-GREEN ALGAE

A. Introductory Studies on the Purity, Stability, and Purification of Anatoxin-a(s)

Andrew M. Dahlem, Ken-Ichi Harada, Nik A. Mahmood, Wayne W. Carmichael
Val R. Beasley

Introduction

The cyanobacteria Anabaena flos-aquae produces potent neurotoxins both in nature and in laboratory culture. One toxin produced by Anabaena is called anatoxin-a(s) and it has been demonstrated to tightly bind and to inhibit cholinesterase. Anatoxin-a(s) remains structurally uncharacterized to date and exerts its toxic effects by only partially understood mechanisms.

Purification of this toxin has been difficult due to the chemical nature of this compound. The toxin is extremely polar, being charged at biological pH and has negligible UV absorption. Anatoxin-a(s) shows limited solubility in polar organic solvents and instability has been reported in several organic solvents by coinvestigators at Wright State University. Since comparatively little was known about the chemical

properties of this compound, and in order to document toxin purity, alternate methods of detection and purification, as well as stability assessment have been under investigation in our laboratory.

Materials and Methods

Thin Layer Chromatography (TLC)

High performance silica gel TLC plates with fluorescent indicator (UV254) were used. Anatoxin-a(s) which was extracted from algae cells and was evaluated to be greater than 90% pure by HPLC with U.V. detection prior to analysis. Samples were applied to high performance TLC plates with glass microcapillary tubes in concentrations ranging from 0.1 to 25 ug. The plates were placed in preequilibrated TLC chambers and the retention of resulting material was observed. A TLC mobile phase of chloroform-methanol-concentrated ammonium hydroxide (30:30:4) was found to provide intermediate retention for compounds present in the purified toxin.

Solvents and Chemicals

All solvents were HPLC grade and all chemicals were ACS grade.

Detection

The developed TLC plates were observed with short wave U.V. light followed by introduction into a glass chamber containing resublimed iodine. Resulting spots observed were immediately marked on TLC plates and the purity of the toxin was subjectively evaluated by comparing toxin amount as estimated by intensity of the spot resulting from iodine vapor with the spots from other compounds present. Methanolic sulfuric acid (30% H_2SO_4 in MeOH) was also used as a spray reagent to visualize non-anatoxin-a(s) contaminants. With sulfuric acid, plates are preheated at 120°C to drive off

iodine then sprayed with reagent and heated for 5-7 minutes at 120°C then visualized under long wave U.V. light.

Toxin Extraction

Purified toxin was extracted by the method of Mahmood (9) prior to TLC purity evaluation or stability assessment. An alternate method for toxin extraction was developed in our laboratories to attempt to provide a normal phase purification scheme of the free base form of the toxin. In this method lyophilized algae cells (1.0 g) are extracted twice with water (100 ml) for two hours at room temperature (21°C). The material is centrifuged at 2,000 rpm for twenty minutes and the supernatant removed. Following the second water extraction both supernatants are combined and evaporated to dryness in a rotary evaporator. The sample is then transferred to an 8.0 ml disposable culture tube with water. Sample is placed on a pre-conditioned (2 ml MeOH-2ml H_2O) C-18 Sep-Pak Cartridge (Waters Associates, Milford, MA) and the residue collected. The cartridge is then rinsed with 4.0 ml of deionized water and this rinse is also collected (toxin is not retained on C-18). The loading eluate and rinse are combined and evaporated to dryness and the residue reconstituted in 30:30:4 (chloroform-methanol-concentrated ammonium hydroxide). The extract is then passed through a Pasteur pipet lined with glass wool and dry packed with 1 g of flash silica. Fractions are collected and monitored by the TLC method described above. Fractions containing toxin are combined and then evaporated to dryness on a rotary evaporator.

Results and Discussion

Impurities as well as apparent hydrolysis products of anatoxin-a(s) have been separated with the TLC method described (Figure 5). The

solvent system provides intermediate retention of anatoxin-a(s) and good separation of toxin from other contaminants present. The detection reagents of iodine vapor and methanolic sulfuric acid are sensitive and detect a side range of compounds which occur as contaminants in purified toxin extracts. Iodine vapor has been routinely used to monitor concentrations of anatoxin-a(s) as low as 0.5 ug and has been the most sensitive method for monitoring low levels of toxin developed to date.

The breakdown and detoxification of anatoxin-a(s) has been correlated with an increase in the concentration of the spot labelled B in Figure 5. This increase in concentration of spot B as determined by intensity of the spot following iodine vapor exposure has been observed under numerous conditions, but most notably after storage in the organic solvents; methanol, ethyl acetate, and acetonitrile.

Spot A was identified as a principal toxic material by passing the purified toxin in TLC mobile phase through a Pasteur pipet lined with glass wool and 1 g of flash silica gel. Spot A was isolated from other compounds present and was injected intraperitoneally in female balb-c mice. Signs typical of anatoxin-a(s) were observed in mice dosed with single isolated compound and death occurred at doses as low as 5 ug/kg.

The alternate method of toxin extraction under investigation and described here has been used to produce small quantities of purified toxin. This method is used to extract the toxin in the free base form and should provide the basis for a normal phase extraction procedure to provide an alternative to reverse phase toxin extraction. We plan to continue collaboration in this area with Dr. Ken-Ichi Harada and his colleagues at Meijo University in Nagoya, Japan. We hope that soon the cooperation of our three research groups will yield analytical methods for extraction and purification of the Anabaena neurotoxins on a scale

sufficient for large scale toxicology testing in animals and structural elucidation of this potent neurotoxin.

B. Introductory Studies on the Purification of Anatoxin-a From Anabaena Flos-Aquae Cells

Introduction

Another potent neurotoxin produced by Anabaena flos-aquae is the depolarizing nicotinic alkaloid anatoxin-a. In addition, methods are available for complete chemical synthesis (10, 11). Anatoxin a has a strong UV absorption and this method of detection as well as alternate methods were investigated initially to provide a method for description of toxin material.

Materials and Methods

Thin Layer Chromatography (TLC)

High performance silica gel chromatography plates with fluorescent indicator (UV 254) were used. Anatoxin-a extract was applied to TLC plates with glass microcapillary tubes. Application was monitored by absorption, by the extract, of short wave UV light. The solvent system which has provided optimum separation of toxin materials is ethylacetate-methanol-water-ammonium hydroxide (170:20:5:3). With this solvent system, the anatoxin-a can be separated from many algae biomolecules and detected with iodoplatinate (platinic chloride) or Dragendorf's spray reagent. Many contaminants are separated with the solvent front. The toxin has an rf of 0.3 by this method.

Toxin Extraction

One gram of lyophilized cell material was extracted with 100 ml of deionized water. The extraction solution was centrifuged at 2,000 rpm for 10 minutes and the supernatant removed. The

supernatant was extracted with 3 x 50 ml of ethyl acetate at neutral pH and the ethyl acetate layers were combined and evaporated. The residue was reconstituted in 0.5 ml of methanol and to 10 ml with water. The toxin solution was passed through preconditioned C-18 cartridges (SEP PAK) and the eluate collected. The cartridge was rinsed with (9:1) water-methanol (4 ml) and this extract was combined with the eluate and evaporated to dryness. The resulting material was monitored by application of the TLC method described for anatoxin-a. Toxin purification was accomplished by silica column chromatography with monitoring of fraction collection by TLC. Fractions containing toxin only were combined and removed for further studies. Small quantities of toxin, which are approximately 75% pure by TLC, have been produced by this method. HPLC with UV detection should provide a valuable tool for final purification.

Results and Discussion

Anatoxin-a is easily detectable by UV absorption or alkaloid locating TLC spray reagents. To date no purified standard exists for comparison of toxin containing extracts to pure toxin material. The TLC methods here should provide a foundation for normal phase toxin extraction of anatoxin-a.

III. FUTURE ANALYTICAL RESEARCH AND PRIORITIES

Our first priority is to continue analytical methods development for the extraction and purification of algal neurotoxins in collaboration with researchers at Wright State University and Meijo University. Algae cells are being continuously produced in culture at Wright State University and, as soon as rapid and efficient extraction methods for algae neurotoxins can be acquired and standardized, we hope to use stored cellular material to rapidly produce pure neurotoxins in sufficient quantity for large scale toxicologic studies.

Another priority is to continue to investigate structure-toxicity relationships of the substituent functional moieties of blue-green algae toxin by subtle synthetic manipulations. Immediate experiments in this area are expected to continue with the two peptide hepatotoxins since they can be purified and produced in sufficient quantities for evaluation at this time.

A third priority is to continue to develop radiolabelled algae toxins through biosynthetic and synthetic means. These labelled toxins will permit development of analytical methods for extraction from biological matrices to confirm animal exposure. The labelled toxins will also be used to characterize the biological fate of algal toxins in animals.

Other priorities include: the development of screening procedures for toxic algae other than mouse bioassay and the establishment of methods to neutralize algae toxins in potable water.

REFERENCES

1. Brooks, W.P. and Codd, G.A. Extraction and purification of toxic peptides from natural blooms and laboratory isolates of the cyanobacterium Microcystis aeruginosa. Letters in Applied Microbiology. (1986), 2, 1-3.

2. Botes, D.P., Kruger, H., and Viljoen, C.C. Isolation and characterization of four toxins from the blue-green alga, Microcystis aeruginosa. Toxicon. 1982 V 20, 6, pp 945-954

3. Siegelman, A.W., Adams, N.H., Stoner, R.D. and Slatkin, D.W. Toxins of Microcystis aeruginosa and their hematological and histopathological effects. In Seafood Toxins, p. 407 (Ragelis, E., Ed.). American Chemical Society Symposium Series 262. Washington, D.C.

4. Krishnamurthy, T., Carmichael, W.W., and Sarver, E.W. Investigations of freshwater cyanobacteria (blue-green algae) toxic peptides. I. Isolation, purification and characterization of peptides from Microcystis aeruginosa and Anabaena flos-aquae. Toxicon. In Press.

5. Botes, D.P., Wessels, P.L., and Kruger, H. Structural Studies on Cyanoginosins-LR, -YR, -YA, and -YM, Peptide Toxins from Microcystis aeruginosa. J Chem. Soc. Perkin Trans I. pp 274-2748, 1985.

6. Matsuo, H., and Morita, L. Improved tritium-labeling for quantitative C-terminal analysis. In Protein Sequence Determination: A Sourcebook of Methods and Techniques, pp. 104-113. (Needleman, S.B., ed.) 1975. Springer-Verlag, New York

7. Gross, E., Subtilin and Nisin: The Chemistry and Biology of Peptides with alpha, B-Unsaturated Amino Acids. In Peptides: Chemistry, Structure, Biology. Proceedings of the Fourth American Peptide Symposium (Walter, R. and Weinhofer, J. eds.) pp. 31-42. Ann Arbor Science Publ., Inc. Ann Arbor, MI.

8. Mahmood, N.A. Neurotoxins from the Cyanobacteria Anabaena flos-aquae NRC 525-17 and Aphanizomenon flos-aquae NH-5. In Ph.D. thesis. Wright State University, Dayton, Ohio. 1985

9. Danheiser, R.L., Morin, J.M., and Salaski, E.J. Efficient total synthesis of (+,-)-anatoxin-a. J. Am. Chem. Soc. 1985. V. 107,26, pp. 8066-73.

10. Petersen, J.S., Fels, G., and Rapoport, H. Chirospecific synthesis of (+) and (-)-anatoxin-a. J. Am. Chem. Soc. 1984. V. 106, 16, pp. 4539-47.

Figure 1 Microcystin extraction procedure used in the laboratories of Dr. Wayne W. Carmichael.

1) 1 GM CELLS + 200 ML 5% BUTANOL-20% METHANOL-75% WATER
STIR 1-3 HRS. AT 4 DEG C.
CENTRIFUGE 100,000 X G - 1 HR AT 4 DEG C.
REPEAT 3 TIMES WITH CELL PELLET

2) COMBINE SUPERNATANTS
REDUCE VOLUME TO 300-350 ML BY AIR DRYING

3) SUPERNATANT PASSED THROUGH ANALYTICHEM BOND ELUTE C-18 COLUMN
ELUTE TOXIC FRACTION WITH 3-5 ML 100% MEOH
REPEAT PROCESS 3-4 TIMES (3-4 X)

4) DRY COMBINED METHANOL EXTRACT WITH NITROGEN
DISSOLVE RESIDUE IN 5 ML WATER
PASS THROUGH 3.0 MICRON MILLIPORE FILTER

5) K-26 PHARMACIA COLUMN (26MM X 80 CM)
WITH 100 GM SEPHADEX G-25
ELUTE IN 5% METHANOL WATER
MONITOR AT 240 NM
TOXIN IS FIRST LARGE PEAK OFF THE COLUMN

6) HPLC-ALTEX C-18 9.4MM X 25CM
0.01 AMMONIUM ACETATE IN 26% ACETONITRILE/WATER
FLOW RATE 3 ML/MIN
MONITOR AT 240 NM

7) LYOPHILIZE TOXIC PEAK
DESALT TOXIN BY HPLC
AS IN STEP 6 - USING 26% ACETONITRILE/WATER

8) STORE TOXIN AT -80°C UNTIL USE

Figure 2 Modified extraction method for microcystin developed at the University of Illinois and used for Monroe Strain.

1) 1 GM CELLS + 200 ML METHANOL
STIR 1-3 HOURS AT 21°C
CENTRIFUGE AT 3,000 x G FOR 10 MINUTES
REMOVE SUPERNATANT

2) EVAPORATE SUPERNATANT IN ROTARY EVAPORATOR TO DRYNESS
REDISSOLVE RESIDUE IN APPROXIMATELY 10 ML OF H_2O

3) RESIDUE PASSED THROUGH PRECONDITIONED ANALYTICHEM BOND ELUTE C-18 COLUMN
C-18 COLUMN WITH TOXIN RINSED WITH APPROXIMATELY 5 UL OF H_2O
ELUTE TOXIN IN 3 ML OF METHANOL
EVAPORATE METHANOLIC TOXIN SOLUTION UNDER NITROGEN STREAM

4) SLURRY PACK SILICA GEL COLUMN WITH $CHCl_3$:MeOH:H_2O
65:35:10 (BOTTOM LAYER)
50 GRAMS OF FLASH SILICA IS PACKED INTO 30 CM x 2.55 CM GLASS COLUMN
TOXIN CONTAINING RESIDUE IS DISSOLVED IN COLUMN MOBILE PHASE AND INTRODUCED INTO COLUMN
THE TOXIN IS ELUTED UNDER ISOCRATIC CONDITIONS AND MONITORED BY TLC

5) SILICA COLUMN TOXIN CONTAINING FRACTIONS ARE COMBINED AND THE ORGANIC SOLVENT EVAPORATED

6) HPLC - C-18 - ALTEX 4.6 MM X 25 CM
METHANOL: .05% TRIFLUOROACETIC ACID IN H_2O (70:30)
FLOW RATE = 2.0 ML/MIN, TOXIN ABSORBS AT 240 MIN

7) COMBINE FRACTIONS CONTAINING TOXIN

8) DESALT WITH KRPTOFIX 221B POLYMER AND KRPTOFIX 222B POLYMER
(E. MERCK, DARMSTADT, FRG)

Figure 3 Tritium incorporation into free alpha-carboxyl residues

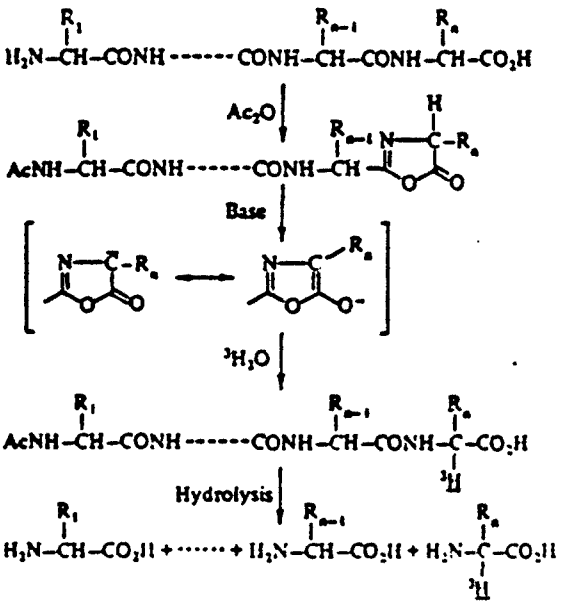
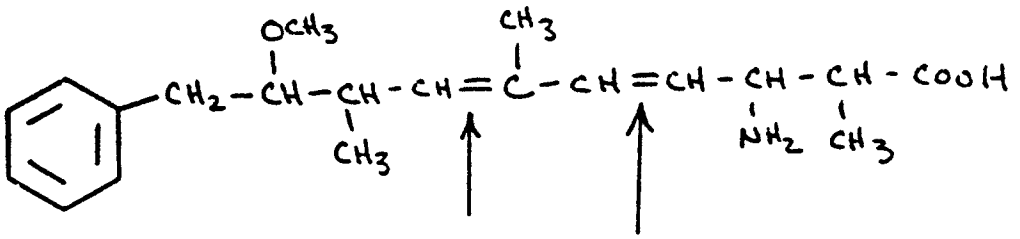


Figure 4 Locations of hydrogen addition in the hydrophobic tail of the ADDA moiety.



3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyl deca-4,6-dienoic acid

Figure 5 Thin layer chromatography of purified anatoxin-a(s) material.

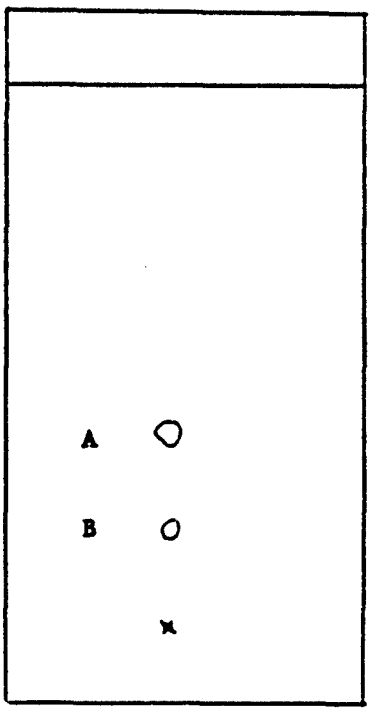


Table 1. Comparison of the toxicity of Nodularia toxin with its hydrogenated derivative.

Dosage	Survival Time	% Liver Weight[1]	% Kidney Weight[2]
Nodularia Toxin			
25 ug/kg			
No. 1	1,630 min[3]	4.4	1.1
No. 2	1,626 min[3]	5.4	1.3
No. 3	1,623 min[3]	3.7	1.3
50 ug/kg			
No. 1	1,621 min[3]	4.2	1.3
No. 2	1,618 min[3]	4.2	1.4
No. 3	1,613 min[3]	4.5	1.2
100 ug/kg			
No. 1	224 min	7.7	1.3
No. 2	127 min	7.9	1.5
No. 3	161 min	8.1	1.4
200 ug/kg			
No. 1	63 min	8.7	1.5
No. 2	125 min	7.8	1.6
No. 3	90 min	8.0	1.5
300 ug/kg			
No. 1	89 min	10.2	1.5
No. 2	84 min	8.8	1.5
No. 3	100 min	8.9	1.6
Control			
No. 1 (1 cc saline)	124 min[3]	5.3	1.4
No. 2 (.75 cc saline)	93 min[3]	4.2	1.3
No. 3 (.50 cc saline)	1,546 min[3]	5.2	1.6
No. 4 (.25 cc saline)	175 min[3]	4.2	1.4

1. Liver weight expressed as percentage of mouse body weight
2. Kidney weight expressed as percentage of mouse body weight
3. Killed by cervical dislocation

Table 1, Continued

Dosage	Survival Time	% Liver Weight[1]	% Kidney Weight[2]
Hydrogenated Nodularia Toxin			
100 ug/kg			
No. 1	1,553 min[3]	4.0	1.2
No. 2	1,556 min[3]	4.6	1.6
No. 3	1,555 min[3]	4.5	1.0
200 ug/kg			
No. 1	1,550 min[3]	5.8	1.2
No. 2	1,553 min[3]	4.2	1.1
No. 3	1,549 min[3]	5.4	1.3
300 ug/kg			
No. 1	1,547 min[3]	6.2	1.5
No. 2	1,542 min[3]	5.9	1.4
No. 3	1,542 min[3]	5.2	1.4
400 ug/kg			
No. 1	1,545 min[3]	6.0	1.2
No. 2	1,540 min[3]	4.5	1.8
No. 3	1,538 min[3]	6.4	1.3
500 ug/kg			
No. 1	177 min	8.7	1.5
No. 2	121 min	8.5	1.7
No. 3	142 min	8.0	1.8
1,000 ug/kg			
No. 1	138 min	9.5	1.7
No. 2	191 min	9.0	1.5
No. 3	141 min	8.9	1.7

1. Liver weight expressed as percentage of mouse body weight
2. Kidney weight expressed as percentage of mouse body weight
3. Killed by cervical dislocation

Annual Progress Report for the Year September 1, 1985 to August 31, 1986

PRELIMINARY STUDIES WITH CYANOBACTERIAL NEUROTOXINS

Bill Cook, Alan Parker, John Dellinger, Karen Harlin, Gregg Lundeen

CONTENTS

1. INTRODUCTION

Preliminary studies during the first year were conducted with two cyanobacterial (Anabaena flos-aquae) neurotoxins anatoxin-a(s), [antx-a(s)] and anatoxin-(a) [antx-a]. The majority of the work was performed with antx-a(s), a known cholinesterase inhibitor, and was aimed at characterizing and evaluating the clinical signs associated with this toxin, determining parameters and end points needed for formal studies, and elucidating the pathophysiologic mechanisms of this toxin.

The structure of antx-a(s) to date is still unknown, therefore, whole blue-green algal cellular material and algal extracts of increasing purity have been used in studies with laboratory animals.

Field cases of anatoxin-a(s), one involving ducks and the other swine, were diagnosed in Illinois during 1986 by the blue-green algae research team at the University of Illinois, working in conjunction with Nik Mahmood, Ed Hyde and the algae research team at Wright State University, Dayton, Ohio.

2. PRELIMINARY STUDIES OF ANATOXIN-A(S) WITH MICE

a. Brain Cholinesterase Activity in Mice Dosed with Anatoxin-a(s), A Neurotoxin Produced by the Freshwater Cyanobacteria, Anabaena flos-aquae NRC 525-17 Compared to Physostigmine, Paraoxon and Pyridostigmine

MICE

Introduction

Anatoxin-a(s), a known cholinesterase inhibitor, was evaluated for possible brain acetlycholinesterase inhibition. The effect of antx-a(s) on brain cholinecterase activity in mice was compared with that of the carbamates physostigmine and pyridostigmine, and the organophosphate, paraoxon.

Materials and Methods

Male, 25-35 gram, Balb/c mice (Sprague Dawley, Indianapolis, IN) were housed in air-conditioned quarters on a 10 hour dark, 14 hour light cycle and were provided feed, Wayne Research Animal Diet, (Rodent Blox, Bemis Co., Inc., Peoria, IL), and water ad libitum.

Groups of 4-7 mice were dosed intraperitoneally (IP) with antx-a(s), physostigmine (Antilerium, physostigmine salicylate, O'Neal, Jones and Feldman, Inc., St. Louis, MO), pyridostigmine (Regonal, pyridostigmine bromide, Organon Inc., West Orange, NJ), paraoxon (Sigma Chemical Co.,

St. Louis, MO), and a control solution comprised of distilled deionized water containing 1% ethanol. Anatoxin-a(s) produced by the method of Mahmood and Carmichael was obtained from W.W. Carmichael (Wright State University, Dayton, OH).[1] Anatoxin-a(s) stored at -20°C prior to use, was brought into solution for dosing with ethanol such that the final dosing solution contained less than 1% ethanol. Paraoxon was stored at a concentration of less than 10 millimolar in dry acetone at -20°C prior to use. The final dosing solution of paraoxon contained less than 1% acetone. Distilled deionized water was used in diluting all toxins for dosing. The control solution was administered in volumes equal to that administered with antx-a(s) on a kilogram weight basis. This volume was the maximum administered on a weight basis with any of the toxins. Doses are shown in Table 1. Injections were made with 1 ml tuberculin syringes having a 25 gauge, 5/8" needle.

One group of mice was dosed with each toxin daily from 9-12:00 AM. Surviving mice were killed at 24 hours by cervical dislocation. Five groups of 4-7 mice were dosed with each toxin, a total of 32 mice with antx-a(s), 35 mice with pyridostigmine, physostigmine and paraoxon, and 20 mice with the control solution.

The entire brain of mice dying prior to 24 hours or killed at 24 hours was removed, weighed, frozen in liquid nitrogen, and stored at -80°C until analyzed for cholinesterase activity. Brain tissue was analyzed for cholinesterase activity by the Modified Ellman Method with the modification of grinding (Broek Tissue Grinder, Fisher Co., Itasca, IL) the entire brain in 20 ml of 1% Triton X (Triton X-100, Sigma Chemical Co., St. Louis, MO).[2] Cholinesterase activity was measured on a Perkin Elmer Lamda 3 UV/VIS Spectrophotometer (Perkin Elmer, Norwalk, CT).

Statistical Analysis

Statistical analysis of the data is incomplete.

Results

The doses of toxins, numbers of mice that died at each dose, and results of brain cholinesterase determinations are presented in Table 1 and 2. Data has not been statistically analyzed, however, there appeared to be no remarkable depression in brain cholinesterase activity in mice that died after intraperitoneal injection of antx-a(s) or the peripheral acting carbamate pyridostigmine. In comparison, the central/peripheral acting carbamate, physostigmine, and the organophosphate, paraoxon did cause brain cholinesterase depression in mice that died before 24 hours. Brain cholinesterase activities in mice dosed with antx-a(s), physostigmine or pyridostigmine and killed at 24 hours after injection did not reveal brain cholinesterase inhibition. In contrast, mice dosed with paraoxon and killed at this time did have depression of brain cholinesterase activity. Clinical signs observed in mice dosed with antx-a(s) (Table 3) included the parasympathetic signs of thick salivation, mucoid lacrimation, urination, and either mucus excretion from the rectum or diarrhea. Nicotinic signs included muscle tremors and fasciculations. The character of respiration changed in antx-a(s) dosed mice and was characterized by a more pronounced respiratory effort with an increased abdominal component. Mice appeared cyanotic prior to death. The animals squinted their eyelids shortly after dosing, but with time squinting disappeared and was replaced in some mice by bulging of the eyes. Clinical signs in animals injected with antx-a(s) persisted longer than the clinical signs observed in mice given either of the carbamates. Decreased movement was generally observed in mice until just prior to

death when clonic seizures occurred. Penile erection was observed at death with the greatest concentrations of antx-a(s) administered.

Discussion

In mice dying or killed 24 hours postdosing with antx-a(s), no remarkable depression of brain cholinesterase activity was observed, suggesting that antx-a(s) did not cross the blood brain barrier. In this regard, antx-a(s) was acting more like the peripheral acting carbamate, pyridostigmine. However, clinical signs in mice dosed with antx-a(s) persisted longer than those observed with the carbamates. The time frame of clinical signs with antx-a(s) was more similar to that observed with the organophosphate, paraoxon. Clinical signs in antx-a(s) dosed mice were consistent with that of a cholinesterase inhibitor and reflected stimulation at muscarinic and nicotinic parasympathetic cholinergic receptors.

b. Preliminary Red Blood Cell (RBC) and Plasma Cholinesterase Reversibility Study in Mice After Intraperitoneal Injection of Anatoxin-a(s).

Introduction

Anatoxin-a(s), a demonstrated irreversible cholinesterase inhibitor in vitro, was evaluated in vivo for reversal of cholinesterase inhibition in mice.[3]

Materials and Methods

Toxin was provided by W. W. Carmichael, Wright State University, Dayton, OH. Fourteen Balb/c mice (Harlan Sprague Dawley, Indianapolis, IN) housed and fed as previously described were injected IP with 200 ug of antx-a(s)/kg body weight (BW). An equivalent volume of distilled water containing 1% ethanol served as a control and was injected IP in 3 mice. Anatoxin-a(s) was mixed into solution with ethanol such that the

final dosing solution contained less than 1% ethanol. Mice were decapitated at 30 minutes, 6 hours and 24 hours postinjection. Blood was collected via a funnel into a 1.9 ml polypropylene micro centrifuge tube containing 15 ul sodium heparin. A fifty ul sample of RBCs obtained after blood was centrifuged at 2,500 RPM for 5 minutes, was lysed with 1.95 ml of 5% Triton X. One hundred ul of this lysate was brought to 10 ml with phosphate pH 8 buffer, and 3 ml of the final solution was analyzed for cholinesterase activity by the Modified Ellman Method. Plasma was analyzed by the Modifided Ellman Method.

Results

Results are presented in Table 4. Mice dosed with antx-a(s) toxin exhibited diarrhea and urination after injection. At 30 minutes, plasma and RBC cholinesterase activities were approximately 27% and 43% of control values, respectively. At 24 hours, plasma cholinesterase activity was still only 50% of control plasma cholinesterase at 24 hours whereas RBC cholinesterase activity had returned to normal.

Discussion

Mouse plasma cholinesterase appeared more sensitive to inhibition by antx-a(s) than mouse RBC cholinesterase. This pattern of cholinesterase inhibition was similar to that observed with organophosphate compounds. Plasma and RBC cholinesterase in mice dosed with antx-a(s) were not rapidly reversible.

3. PRELIMINARY STUDIES OF ANATOXIN-A(S) AND ANATOXIN-A WITH RATS

Introduction

Rats were dosed orally and intraperitoneally with blue-green algae neurotoxins to assess the susceptibility of this species to two Anabaena flos-aquae neurotoxins anatoxin-a(s) and anatoxin-a, to characterize clinical signs by these routes of administration and to evaluate electroencephalographic alterations [antx-a(s) only].

Materials and Methods

Fischer 344 rats (Sprague Dawley, Indianapolis, Indiana) weighing 200-350 grams were gavaged via a 7.6 cm stainless steel gavage needle with lyophilized Anabaena flos-aquae cells containing antx-a(s) (W.W. Carmichael, Wright State University, Dayton, Ohio). Rats were dosed at 0.1, 0.5, 1.0 and 2.0 gm of blue-green algae/kg BW. Algae was mixed in distilled water in final dosing volumes of less than 4.1 ml.

One Fisher 344 rat was gavaged via a 7.6 cm stainless steel gavage needle with 0.5 gm and 2 gm of antx-a lyophilized blue-green algae cellular material containing antx-a (W.W. Carmichael, Wright State University, Dayton, Ohio)/kg BW. Lyophilized algae was mixed in distilled water with a final dosing volume of 3.7 ml.

Two Fisher rats as described above were dosed intraperitoneally with lethal doses (1.25-3.4 ml) of "semipurified" antx-a(s). "Semipurified" antx-a(s) was extracted from lyophilized algae using the method of Carmichael and Mahmood, except for omission of ultracentrifugation and gel filtration steps so that the concentrated, acidified algal extract was placed directly into C-18 Sep Pak cartridges. In this method, five grams of cellular material was extracted with 250 ml of ethanol and brought up in a final volume of 50 ml of deionized water. An electroencephalograph machine (Grass 7/8 physiograph/EEG, Grass Instrument Co., Quincy, MA) was used to monitor electrical activity in the brain of rats.

Results

No clinical signs were observed in rats gavaged with lyophilized algal cells containing antx-a(s) at any dose. However, "semipurified" antx-a(s) was lethal in rats by the IP route, and the clinical signs observed in these rats are listed in Table 5.

Electroencephalographic alterations observed in the brain of one rat dosed IP with "semipurified" antx-a(s) included crude spike activity that preceded seizure activity and dysrhythmic activity (slow wave activity) at times other than seizures.

Clinical signs observed in the rat dosed with 2 grams of algae containing antx-a/kg BW consisted of ataxia, muscle tremors, dropping of the head, recumbency, lack of tail movement, intermittant hopping movement due to contractions of the rear legs, dyspnea, head pressing, and a cool external body temperature.

Discussion

The rat was resistant to antx-a(s) toxicosis after oral administration of lyophilized algae. The electroencephalographic alterations and clinically observed narcotization observed in one rat after IP administration of algae containing antx-a(s) may indicate a central effect of antx-a(s) on the brain of the rat. The possibility of EEG alterations being due to anoxia could not to be ruled out. Rats were susceptible to toxicosis after oral administration of lyophilized algae containing antx-a.

4. PRELIMINARY STUDIES OF ANATOXIN-A(S) WITH CHICKENS

Introduction

Chickens were utilized for preliminary evaluation of possible delayed neurotoxicity with antx-a(s).

Materials and Methods

A 1.2 kg pullet was dosed with 3.0 and 6.0 ml of crude extract of lyophilized blue-green algae material on consecutive days. A second 2 year old, 2.1 kg white Leghorn laying hen was dosed via a jugular catheter (21 x 3/4" butterfly infusion set) with 5.0 ml of the extract. Crude extract was prepared by mixing 1.5 grams of lyophilized algae in 18

ml of isotonic NaCl, sonication for 10 minutes, centrifugation at 3,000 RPM for 10 minutes and filtration through a 0.45 uM filter. Chickens where observed daily for signs of delayed neurotoxicity.

Results

The first chicken died within 24 hours of the second dosing in the acute stage of antx-a(s) toxicosis without time to possibly develop any signs of delayed neurotoxicity. Clinical signs observed in the second chicken after dosing included salivation, diarrhea, muscle tremors, ataxia, reluctance to move and drooping of the head. Clinical signs abated by 18 hours postdosing. After dosing this chicken lost weight for 9 consecutive days and did not lay eggs for 18 days. The chicken was observed for 35 days with no clinical signs of delayed neurotoxicity.

5. PRELIMINARY STUDIES OF ANATOXIN-A(S) WITH PIGS

Introduction

Preliminary studies utilized pigs to examine the toxicity, clinical signs, and possible pathophysiologic mechanisms of antx-a(s).

Materials and Methods

Crossbred gilts 12-40 kg (Veterinary Research Farm, University of Illinois, Urbana) were fed a 16% protein, corn-soybean base diet and provided water ad libitum prior to dosing studies.

Twelve hours prior to surgical procedures, pigs were taken off feed. Animals were anesthetized via inhalation of halothane (Fluothane using a Fraser Harlake Halothane Machine, Ohmeda Co., Orchard Park, NY). Jugular and carotid catheters (16 gauge Tygon Tubing, Microbore, A. Daigger and Co., Chicago, IL) were inserted via an incision in the left jugular furrow. Insertion of aortic and left atrial catheters was by surgical incision behind and with partial removal of the 4th rib.

Catheter ports were tunneled to subcutaneous sites over the back where they were later exteriorized on the day of dosing with surgical excision under lidocaine induced local anesthesia. Anatoxin-a(s) was administered in successive doses intravenously in 3 pigs. One pig was dosed with 10 ml of "semipurified" antx-a(s) via the ear vein using a 22 gauge butterfly catheter.

Blood pressure and EKG recordings were made on a Gilson physiograph (Gilson Electronics, Inc., Middleton, WI). Blood pressure readings were made with Gould Statham pressure transducers (Gould Statham Instruments, Inc., Hato Ray, Puerto Rico). Blood gas determinations on heparinized blood samples was determined with an IL 813 blood gas machine (Instrumentation Laboratories, Lexington, MA).

Plasma and brain cholinesterase activities were determined by the Modified Ellman Method. One hundred ul of red blood cells obtained by centrifuging whole blood EDTA containing at 2,500 RPM for 5 minutes, were lysed in 1.9 ml of 5% Triton X. Two hundred ml of the lysate was brought to 10 ml with phosphate pH8 buffer, and 3 ml of this solution was used for cholinesterase analysis.

Results

Clinical signs observed during intravenous dosing studies in pigs are presented in Table 6. Times of dosing, amounts of toxin administered, RBC and plasma cholinesterase activities, and blood gas results are presented in Tables 7 to 9. No marked seizure activity or evidence of miosis were observed in any pigs. Blood gas changes in general included increases in pCO_2 and marked decreases in pO_2 and blood pH. Blood gas changes reflected death due to anoxia.

In general, evaluation of electrocardiograms has not revealed changes that would be expected with a classic cholinesterase inhibitor,

except for a mild bradycardia in one pig. Evaluation of electroencephalograph recordings have revealed no alterations consistent with a direct affect of antx-a(s) on the brain in 3 swine that died after successive intravenous doses of antx-a(s). Electromyograph evaluation of muscle tremors indicated tremors were due to motor unit action potentials (lower motor neuron origin) and did not apparently originate from the muscle itself. The EMG findings were compatible with those of a cholinesterase inhibitor.

Blood pressure readings have only been recorded from 1 pig, (Table 9a) but revealed a marked, but temporary increase in pulmonary artery pressure after toxin administration (Figure 1). A transient decrease (30 sec.) in aortic mean blood pressure occurred simultaneously each time pulmonary artery pressure increased. A transient tachycardia was observed coincident with the drop in aortic blood pressure. Pulmonary arteriolar vasoconstriction is a mechanism that could account for some of the blood pressure changes.

A 15 kg pig was administered 10 ml of "semipurified" antx-a(s) via the ear vein over 10 seconds. The pig died in 18 minutes after exhibiting ataxia, salivation and tremors. The skin of the pig became brightly erythematous after dosing suggesting peripheral vasodilation. Probable sympathetic stimulation induced by handling made the erythema disappear. No seizures or clinical signs that could be directly attributed to central nervous system dysfunction were observed.

Analysis of brain cholinesterase activity in the 4 swine that died of antx-a(s) have revealed cholinesterase activity within normal ranges. Progressive, cholinesterase inhibition was observed in plasma and RBC with successive toxin administration. In general, RBC cholinesterase was inhibited more than plasma after antx-a(s) administration.

Discussion

Neurologic examinations, EEG recordings and brain cholinesterase activity determinations in pigs suggested that antx-a(s) doses not cross the blood brain barrier in pigs. The cause of death in pigs appears to be anoxia and acidosis secondary to cholinesterase inhibition and respiratory paralysis. Recent work has revealed alterations in pulmonary artery blood pressure in 1 pig, and this finding warrants confirmation in future studies.

Table 1. Doses of "semipurified" anatoxin-a(s), physostigmine, pyridostigmine and paraoxon injected intraperitoneally into mi -. the number of mice that died before 24 hours and the nu er of mice dosed.

Toxin	Dose	Died before 24 hours/number of mice dosed
Anatoxin-a(s)	225.0 ug/kg	2/7
"	228.75 ug/kg	3/7
"	232.5 ug/kg	7/7
"	240.0 ug/kg	5/7
"	700.0 ug/kg	4/4
Physostigmine	650.0 ug/kg	1/7
"	700.0 ug/kg	0/7
"	725.0 ug/kg	4/7
"	750.0 ug/kg	5/7
"	950.0 ug/kg	7/7
Pyridostigmine	1,150 ug/kg	0/7
"	1,450 ug/kg	0/7
"	2,100 ug/kg	3/7
"	2,150 ug/kg	3/7
"	2,250 ug/kg	5/7
Paraoxon	1,000 ug/kg	0/7
"	1,250 ug/kg	3/7
"	1,275 ug/kg	4/7
"	1,312 ug/kg	7/7
"	1,375 ug/kg	6/7

Table 2. Mean brain cholinesterase activities of mice that died prior to 24 hours or killed at 24 hours after intraperitoneal injection with anatoxin-a(s), physostigmine, pyridostigmine paraoxon or control solution.

TOXIN	STATUS		CHOLINESTERASE ACTIVITY (uM/g/min)	
	Died	Killed at 24 hours	Died	Killed
Anatoxin-a(s)	n=22	n=12	10.59	10.42
Anatoxin-a(s) highest dose	n=4	—	9.85	—
Physostigmine	n=17	n=18	1.87	10.55
Pyridostigmine	n=11	n=24	10.04	10.29
Paraoxon	n=20	n=15	0.11	3.81
Control	—	n=20	—	10.38

Table 3. Clinical signs categorized by suspected site of action in mice injected intraperitoneally with anatoxin-a(s).

Postganglionic neuroeffector sites		Ganglionic (suspected)	
Acetylcholine mediated		Acetylcholine mediated	Other
Muscarinic	Nicotinic	Parasympathetic	
Thick salivation	Muscle tremors	Penile erection	Ataxia
Mucus secretion from rectum	Muscle fasciculation		Decreased movement
	Dyspnea		Jaw movements
Diarrhea	Increased abdominal component to respiration		Squinting of eyes
Mucus lacrimation			Bulging of eyes
Urination			Cyanosis
Rectal sphincter dilation			Clonic seizures

Table 4. Reversibility of plasma and red blood cell cholinesterase activity in mice dosed intraperitoneally with anatoxin-a(s) and control.

Time	Anatoxin-a(s)					Control		
		Cholinesterase*					Cholinesterase*	
	No. of Mice	Plasma	% Inhibition	RBC	% Inhibition	No. of Mice	Plasma	RBC
30 min	n = 4	0.51±0.15	73	0.26±0.10	56.7	n = 1	1.85	0.60
6 hours	n = 4	0.93±0.176	50.8	0.46±0.12	23.3	n = 1	2.33	0.56
24 hours	n = 6	0.95±0.21	49.7	0.60±0.14	0.0	n = 1	1.48	0.50
Average of control mice injected						n = 3	1.89± 0.35	0.60± 0.04

*Units in uM/l/min

Table 5. Clinical signs in rats after intraperitoneal injection of lethal doses of anatoxin-a(s)

Salivation
Chromodacryorrhea
Mucus discharge from the rectum
Rectal sphincter dilation
Muscle tremors and fasciculations
Increased respiratory effort
Dyspnea
Increased abdominal component of respiration
Decreased rectal temperature
Cool external body temperature
Ataxia
Decreased righting ability
Generally decreased movement
Intermittent leaping
Narcosis
Jaw movements prior to death
Cyanosis
Clonic seizures

Table 6. Clinical signs in 3 pigs after intravenous administration of "semipurified" anatoxin-a(s).

Narcosis
Salivation
Mucoid nasal discharge
Muscle fasciculations
Muscle tremors
Rectal sphincter dilation
Mucus secretion from rectum
Diarrhea
Ataxia
Bruxism
Increased abdominal component to respiration
Dyspnea
Decreased nasal capillary refill time
Nystagmus
Cyanosis
Respiratory paralysis

Table 7a. Plasma and RBC cholinesterase activity and blood gas determinations in a 35.9 kg pig (Pig # 1) after successive doses of "semipurified" anatoxin-a(s)

Time (min)		Dose (ml)	Cholinesterase (uM/l/min) Plasma	Percent Inhibition	RBC	Percent Inhibition
Predose:	0	0	0.343	0.0	2.68	0.0
	0	0.5	—	—	—	—
	15	2.0	—	—	—	—
	30	4.0	—	—	—	—
	45	4.0	—	—	—	—
	47	—	0.095	72.3	0.65	62.7
	60	8.0	—	—	—	—
	75	—	0.098	71.4	0.037	98.6
	77	—	0.086	74.9	0.098	96.3

Table 7b. Blood gas determinations in a 35.9 kg pig (Pig # 1) after successive doses of "semipurified" anatoxin-a(s)

Time (min)		pH	pCO_2	pO_2	Hct	Hb	Temp (°C)
predose:	0	7.448	36.1	99.7	35	11	40.0
	47	7.441	33.1	99.4	30	10	40.0
	75	7.416	38.1	90.87	37	12	40.0
	77	7.259	34.4	80.4	41	12.5	40.0

Table 8. Plasma and RBC cholinesterase activities and blood gas values of a 12.27 kg pig (Pig #2) after successive doses of "semipurified" anatoxin-a(s).

Toxin (ml)	Time (hr)		Blood Gas (mm Hg)			Cholinesterase (uM/L/min)			
			pH	pCO2	pO2	Temp*	Plasma	%Inh.	RBC	%Inh.
0	predose	A	7.48	32.8	82.7	39.5	0.306		2.12	
		V	7.44	36.9	35.9					
0.33	0	A	7.5	27.4	90.7	39.9	—		—	
		V	7.4	30.9	33.4					
0.33	0.5	A	7.5	30.1	88.7	40.2	0.282	7.8	1.67	21.2
		V	7.4	35.0	36.4					
0.33	1.0	A	7.5	27.3	87.1	40.1	0.245	19.9	1.61	21.2
		V	7.4	38.6	37.9					
0.33	1.5	A	7.5	34.0	76.8	40.0	0.221	27.8	1.23	42.0
		V	7.4	37.0	31.7					
0.33	2.0	A	7.5	29.2	88.7	40.1	0.172	43.8	.65	69.3
		V	7.4	37.6	36.4					
0.66	2.5	A	7.5	33.7	91.4	40.	0.110	64.1	.36	83.0
		V	7.4	35.6	34.9					
0.66	3.0	A	7.4	35.7	84.2	40.3	0.086	71.9	.17	92.0
		V	7.4	38.6	34.9					
0.66	3.5	A	7.5	31.5	86.7	40.3	0.049	84.0	.04	98.1
		V	7.4	36.0	32.7					
0.66	4.0	A	7.5	32.3	85.8	40.5	0.025	91.8	0	100
		V	7.4	40.2	29.3					
0.66	4.5	A	7.4	36.2	67.6	40.8	0.025	91.8	0	100
		V	7.4	40.2	33.4					
	4.8	A	7.3	46.6	36.5	40.8	0.025	91.8	0	100
		V	7.1	58.0	15.2					
0.66	5.0	A	7.1	49.0	38.5	41.0	0.025	91.8	0	100
		V	7.0	60.3	8.9					
	5.2	A	6.8	83.9	25.6	41.0	0.025	91.8	0	100
		V	6.8	85.1	6.8					

Total toxin = 5.66ml

A = Arterial
V = Venous
* = (°C)

%Inh. = Percent Inhibition
— = Not determined

Table 9a. Left atrial, aortic, and pulmonary artery mean blood pressure, and heart rate in a 20 kg pig (# 3) administered successive doses of semipurified anatoxin-a(s)

Time Dosed	Route* and Dose	Time of Measurement	Mean Blood Pressure (mmHg) Left Atrial	Aortic	Pulmonary Art.	Heart Rate (Beats/minute)
0		Predose	8	106	22	150
0	A	0-5 min postdose	8	50	44	168
30 min,	B	0-5 min predose	8	112	22	108
		0-5 min postdose	10	54	60	200
60 min,	C	0-5 min predose	10	110	22	108
		0-5 min postdose	10	90	32	120
90 min,	C	0-5 min predose	8	110	23	120
		0-5 min postdose	8	70	45	140
120 min,	C	0-5 min predose	8	104	24	—
		0-5 min postdose	15	50	40	—

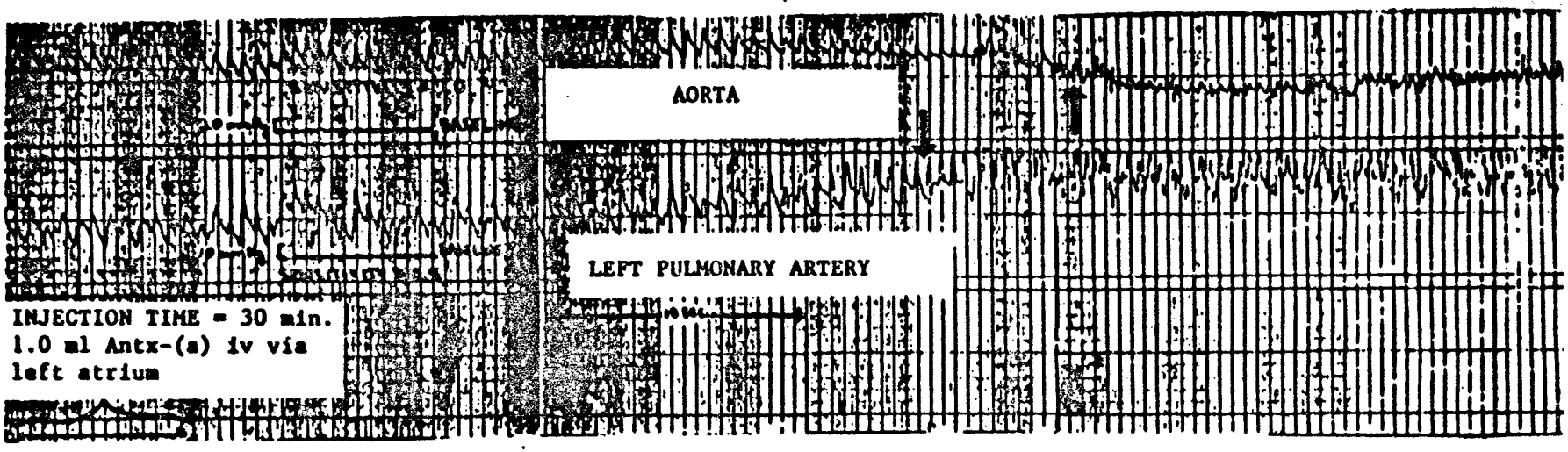
*A = Pulmonary artery injection of 1 ml of "semipurified" anatoxin-a(s) over 5 seconds.
B = Left atrium injection of 1 ml of "semipurified" anatoxin-a(s) over 5 seconds.
C = Left atrium injection of 1 ml of "semipurified" anatoxin-a(s) over 1 minute.
Total toxin administered = 5.0 ml

Table 5b. Plasma and RBC cholinesterase determinations, blood gas and blood pH of a pig (# 3) administered successive doses of "semipurified" anatoxin-a(s)

	Cholinesterase (uM/l/min)				Arterial blood Gases (mmHg)		Blood pH
Time	Plasma	% Inhibition	RBC	% Inhibition	PCO_2	PO_2	
Predose	0.27	0.0	1.81	0.0	40.7	93.8	7.4
0.5 hr.	0.14	48.1	0.72	60.2	37.7	94.7	7.4
1 hr.	0.09	66.7	0.61	66.3	36.0	94.7	7.4
1.5 hr.	0.06	77.8	0.06	96.7	36.2	94.4	7.4
1.75 hr.	—	—	—	—	38.4	81.6	7.4
2.0 hr.	0.06	77.8	0.03	98.3	41.7	78.9	7.4
2.1 hr.	—	—	—	—	39.4	55.2	7.3

— = Not run

Figure 1



Observed increase in left pulmonary artery blood pressure and decrease in aortic artery blood pressure after left atrial administration of 1.0 ml of "semipurified" antx-a(s) in a pig.

(Arrows indicate areas of increasing pulmonary artery blood pressure and decreasing aortic artery blood pressure).

REFERENCES

1. Carmichael WW, Mahmood NA: Toxins from Freshwater Cyanobacteria. Seafood Toxins, E. Raegelis Ed., 1984 ACS Symposium Series 262, ACS Publications, Washington, D.C.

2. Ellman GE, Courtney KD, Andres V, et al: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1916:7, 88-95.

3. Mahmood NA. Personal Communication.

6. FIELD CASES OF ANATOXIN-A(S)

a. Field Toxicosis in Ducks due to Anatoxin-a(s), a Neurotoxin Produced by the Freshwater Cyanobacteria Anabaena flos-aquae, and Studies with Algal Bloom Material Containing Anatoxin-a(s) in Mice, Ducks, Pigs, and Cattle

Introduction

Toxic freshwater blue-green algae (cyanobacteria) are found world wide. The most commonly reported genera of blue-green algae associated with toxic blooms have been Anabaena, Microcystis, and Aphanizomenon, Oscillatoria. Toxins from these algae have been responsible for toxicoses in domestic and wild animals in several areas of the world. Toxins of freshwater blue-green algae have been identified as neurotoxins, hepatotoxins, and toxins causing gastroenteritis and dermatitis. Structurally, these toxins are alkaloids, polypeptides or pteridines that have been partially or completely identified.

Neurotoxins have been isolated from strains of the freshwater blue-green algae Anabaena flos-aquae and Aphanizomenon flos-aquae. Known alkaloid neurotoxins produced by Aphanizomenon, referred to as Aphanitoxins I and II are identical to neosaxitoxin and saxitoxin respectively. Anabaena flos-aquae is believed to produce four to six physiologically distinct neurotoxins, termed anatoxins, which have been designated as anatoxins (antx) a, b, c, d, a(s) and b(s). Designations were based on opisthrotonus, salivation and survival time. According to this designation, antx-a(s) and b(s) caused clinical signs similar to those of antx-a and antx b, but also caused salivation in laboratory animals, and were therefore designated with the "s".

Only antx-a, an alkaloid, has been characterized structurally. Anatoxin-a and a(s) have had their physiologic effects studied. Anatoxin-a is a potent irreversible nicotinic agonist that causes

postsynaptic neuromuscular blockade by reacting specifically with the acetylcholine postsynaptic receptor, and may also have presynaptic activity accounting for an observed increase in presynaptic miniature end plate potentials. Anatoxin-a(s) is an "irreversible acetylcholinesterase inhibitor" that produces excessive cholinergic stimulation. Anabaena flos-aquae clone NRC-525-17, isolated from a bloom in Buffalo Pound, Saskatchewan, Canada in 1965 has been the sole source of algal material containing antx-a(s) for research in mice, rats and chickens. The bloom composition was 90% Anabaena flos-aquae and 10% Aphanizomenon flos-aquae. Over twenty dogs and a few calves were reported to have been poisoned during 1960-1970 and 1965, respectively, by blooms in this lake. The present report describes the clinical signs, conditions, and confirmation of a natural outbreak of Anabaena flos-aquae [antx-a(s)] toxicosis in ducks in Illinois, and experimental dosing studies with toxic bloom material in mice, ducks, cattle, and pigs.

Case Description

Sudden death in 5 Muscovy ducks, and clinical signs of severe ataxia, frequent sternal and lateral recumbency, and wing paralysis in two other Muscovy ducks were associated with a blue-green algal bloom in east-central Illinois. Death and clinical signs occurred over 5 days in late July, 1986. Ducks were found dead in the water of a small farm pond on mornings after they were observed to be normal the night before. Initially, one morning, four ducks were found dead after a mild rain storm the night before. All four of these ducks and a water sample were submitted to the University of Illinois Veterinary Diagnostic Laboratory (UI-VDL) at Urbana. A fifth duck was found dead in the pond 2 days later. At this time clinical signs were observed in 2 other ducks, one of which was immediately submitted to the UI-VDL for necropsy along with

a second water sample. The other duck recovered and was clinically normal after 60 hours.

Nineteen ducks, 15 Muscovies and 4 Mallards had utilized this pond. Twelve Muscovy ducks and one Mallard had hatched this year and were 5 months old. Only younger Muscovy ducks were affected. Most ducks were removed from the pond after the initial death loss. One Muscovy duck that remained on the pond for 4 days afterwards showed no clinical signs. Two horses were not affected that drank from the pond 12 hours before the first ducks were found dead. No deaths were observed in frogs swimming in the pond during the algal bloom.

Green algae was observed in the pond for 1-2 weeks prior to deaths and, when deaths had occurred, wind had concentrated surface algae against the shore. Water in the pond was observed to turn blue after deaths occurred. The pond was constructed 3 years ago and was 7.3 meters deep and only 15 meters in diameter. A drain designed to carry runoff from an adjacent field emptied into the pond.

Histopathologic examination was conducted on tissues from 5 necropsied ducks. Brain cholinesterase activities were determined on the brain tissue of 1 duck found dead on the farm and 1 clinically affected duck submitted live for necropsy.

Algal bloom material was collected 5 days after the initial death loss in the ducks, concentrated by filtration through cheese cloth and refrigerated at 4°C in a gauze covered 22 liter bottle for future dosing studies.

Materials and Methods

Six, 6 month old, 2-3 kg Muscovy ducks from a different farm were housed in a 4 m x 4 m stall, fed 0.5 kg of Purina Duck Chow (Growena L Duck Chow, Purina Co., St. Louis, MO) twice daily and provided water ad

libitum. Four birds were dosed with 22 ml of concentrated algae per kg BW. Two birds serving as controls were dosed at 22 ml of distilled water/kg body weight. Ducks were dosed at 0, 24, 48, 72 and 75 hours by intracrop intubation with a 10 French, 36 cm polypropylene catheter (Sovereign, Monoject, St. Louis, MO 63103) and a 50 cc syringe. Prior to dosing and again shortly before death, birds were bled via the wing vein using a heparinized 1 cc tuberculin syringe and a 26 gauge, 5/8" needle. A seventh 6 month old, 2-3 kg Muscovy duck from the affected farm had been dosed at 22 ml of concentrated algae/kg BW at 0, 24,48, 72 and 98 hours. Plasma was analyzed for cholinesterase activity by the Modified Ellman Method. Ducks surviving 78 hours were killed by decapitation and exsanguination. Brain, lung and breast muscle tissue were analyzed by the Modified Ellman Method.

Algae Identification, Toxin Isolation, Mouse Bioassay (Wright State).

Prior to dosing studies, algae was sent to Wright State University for light and scanning electron microscopic algae identification, high pressure liquid chromatographic (HPLC) analysis (Beckman 340 HPLC), mouse LD_{50} bioassay estimation on HPLC purified antx-a(s), and in vitro cholinesterase analysis. High pressure liquid chromatographic analysis was done on an Altex CN Column (4.9 mm x 0.15 cm) 80/20 (10mM NH_4Ch_3COO/1mM Ch_3COOH at 1.5 ml/min, 230 lambe at 0.10 OD)]. The 2 hour LD_{50} determination was made on 5 groups of 5 mice (ICR Swiss, 19 $\pm$ 1 gram) with a constant IP injection volume of 0.5 ml at doses of 25, 50, 100 and 200 ug of antx-a(s)/kg body weight (BW). A second algae sample was sent to Wright State University for additional mouse toxicity testing after these dosing studies to determine if any significant change in toxicity had occurred over the course of the studies. Assessment of in

vitro inhibition of electric eel acetycholinesterase (Sigma Chemical Co., St. Louis, MO) was assayed by the Modified Ellman Method. Algae was analyzed for organophosphates and carbamates by gas liquid chromatography.

Repeated Oral Dosing of Mice (UI).

Male Swiss Webster mice (Sprague Dawley, Indianapolis, Indiana) weighing 38 ± 4 grams were gavaged daily with 20 ml of concentrated blue-green algae at 22ml/kg BW for 5 days and decapitated 3 hours after the last dosing. Whole blood, plasma, RBC, lung and diaphragm cholinesterase activities were determined. Whole blood and plasma cholinesterase activities were analyzed by the Modified Ellman Method. Fifty ul of RBC, obtained after centrifuging blood at 2,500 RPM for 5 minutes, was lysed with 0.95 ml of 5% Triton X. One hundred ul of this lysate was brought to 10 ml with phosphate pH 8 buffer, and 3 ml of this solution was analyzed for cholinesterase activity by the Modified Ellman Method. Changes in the Modified Ellman Method for analysis of lung and diaphragm included using entire organs, grinding tissues in 10 and 5 ml of phosphate pH 8 buffer respectively, centrifugation at 2,000 RPM for 10 minutes, and addition of 100 ul of supernatant to 2.5 ml of phosphate pH 8 buffer for analysis.

Mouse Intraperitoneal Bioassay (UI).

Four male Swiss Webster mice (36.0 ± 1 gm) were injected intraperitoneally (IP) via a 25 gauge 5/8" needle with 0.25, 0.5 or 1.0 ml of an extract of pond water containing antx a-(s) or 1.0 ml of water for a control. The extract was a supernatant from pond water containing algae that was frozen for 12 hours, thawed, and centrifuged at 10,000 RPM for 10 minutes. Survival time, liver and kidney as a percent of body weight and brain, lung, diaphragm, whole blood and plasma cholinesterase

activities were determined. Four mice (26 + 2.5 gm) were injected with either 0.05 ml, 0.125 ml, 0.2 ml of the extract or 0.2 ml water for a control and decapitated just prior to death (10-15 minutes after dosing). Whole blood and plasma cholinestearse activity, and liver and kidney weights as a percent of body weight were determined.

Intraruminal Administration to a Calf

A 145 kg, 4 month old ruminating crossbred beef steer was housed in a 5 m x 5 m box stall, fed alfalfa hay twice daily and provided water ad libitum prior to dosing. Carotid and jugular catheters (16 gauge Tygon Tubing, Microbore, A. Daigger and Co., Chicago, IL) were inserted via an incision in the left jugular furrow under glycerol guiacolate, xylazine and ketamine anesthesia. Catheter ports were tunneled subcutaneously and exteriorized the first day of dosing under local lidocaine anesthesia. The steer was held off feed and all bedding was removed from 12 hours prior to the first intraruminal dosing until 24 hours after the second intraruminal dosing. The steer was dosed using a stomach tube and bilge pump with 22 ml of concentrated algal material/kg BW followed 24 hours later by 35 ml of concentrated algae/kg BW. Forty-eight hours after the second intraruminal dose the steer was dosed intraperitoneally with 300 ml of algae supernatant. Extract was supernatant from concentrated algae frozen for 12 hours and centrifuged at 15,000 RPM for 10 minutes. Intraperitoneal injection was made via the left paralumber fossa with a 12 gauge trocar under local lidocaine anesthesia. The steer was redosed intraperitoneally 35 minutes later with 250 ml of supernatant. EDTA blood was spun at 2,500 RPM for 5 minutes for plasma and RBC cholinesterase activities. Plasma cholinesterase activity was analyzed by the Modified Ellman Method. Red blood cell cholinesterase activity was determined by lysing 100 ml of RBC in 1.9 ml of 5% Triton X, diluting 200 ml of the

lysate to 10 ml with phosphate pH 8 buffer and using 3 ml for assay in a Lamda 3 UV/VIS spectrophotometer (Perkin Elmer, Norwalk, CT). Brain, lung and diaphragm tissue for cholinesterase activities were frozen in liquid nitrogen and ground in a Waring blender. Two hundred ug samples of tissues were analyzed for cholinesterase activity by the Modified Ellman Method. Lung, diaphragm and brain cholinesterase activities were compared with historical controls.

Intragastric Administration to Swine.

Three 14-17.5 kg swine were fed a 16% protein, corn soybean diet twice daily and water ad libitum. Pigs were administered a preanesthetic dose of atropine and anesthetized with halothane (fluothane using a Fraser Harlake Anesthetic Machine, Ohmeda Co., Orchard Park, NY) for surgical placement of carotid and jugular catheters (16 gauge Tygon Tubing, Microbore, A. Daigger and Co., Chicago, IL) by an incision over the left jugular furrow. Catheter ports were tunneled to a subcutaneous site over the back where they were exteriorized on the day of dosing by surgical excision under lidocaine induced local anesthesia. All pigs were held off feed for 12 hours prior to dosing. Two pigs were gavaged via stomach tube with 22 ml of concentrated blue-green algal material/kg BW. A third pig served as a control and was gavaged with 22 ml of distilled water/kg BW. The stomach tube in each pig was flushed in situ with 50 ml of distilled water after dosing.

Blood gases were determined on a IL 813 Blood Gas Machine (Instrumentation Laboratories, Lexington, MA). Blood, plasma, RBC, lung, brain and diaphragm cholinesterase activities were analyzed as described for the steer above.

One of the pigs given the algae was treated with 0.5 mg of atropine [(Atropine sulphate, Professional Veterinary Laboratories, Minneapolis,

IM)/kg BW (1/4 IV and 3/4 IM)], and 2.2 gm of activated charcoal (Superchar-Vet, Gulf Biosystems, Inc., Dallas, TX)/kg BW intragastrically by stomach tube when clinical signs (salivation) were observed. The control pig was killed by electrocution and exsanguination at the time that the non-treated algae dosed pig died.

Brain, lung, liver, kidney, diaphragm or breast muscle, spleen, pancreas, heart adrenal, and gastrointestinal tissues of ducks, the calf and pigs were taken at necropsy, fixed in 10% buffered formalin, stained with hematoxylin and eosin and sectioned at 6 microns for histopathologic examination.

Results

Dosed Ducks (UI).

Clinical signs observed in orally dosed ducks are presented in Table 1. No miosis was observed. Ducks could appear relatively normal until stressed at which time some would become ataxic, recumbent, and have leg and wing paresis or tonic seizures. Ducks sought out and drank water after dosing. Three ducks died; one died after the second dose, one after the fourth dose and one after the fifth dose. Plasma cholinesterase activity was inhibited just prior to death of ducks compared to predosing levels (Table 2). Cholinesterase determinations on lung and diaphragm tissue revealed cholinesterase depression, but brain cholinesterase activity did not appear inhibited.

Results from Wright State

Algae was identified by light microscopic and scanning electron microscopy as Anabaena flos-aquae. High pressure liquid chromatographic examination of algae extracts revealed a compound with the same retention time as a standard of antx-a(s) (Figure 1). The LD_{50} of HPLC purified antx-a(s) was between 25 and 50 ug of antx-a(s) per kg BW in mice. The

toxicity was similar to that observed for antx-a(s) previously obtained. Mice in the LD_{50} study displayed salivation, lacrimation, urination, muscle fasciculations, respiratory distress and convulsions. Mouse bioassay revealed no remarkable change in the toxicity of algal material over the course of dosing studies. In vitro electric eel cholinesterase assay of toxin revealed cholinesterase depression comparable to that observed with standards of antx-a(s) (Table 3).

Results from UI

Doses, organ weights and tissue cholinesterase determinations from oral and IP dosing studies in mice are presented in tables 4-6. Mice dosed orally demonstrated no clinical signs of antx-a(s) toxicosis, and whole blood, RBC, plasma, lung and diaphragm cholinesterase activities in these mice were not inhibited when compared to controls. Intraperitoneally injected mice had clinical signs of salivation, mucoid lacrimation, diarrhea and clonic convulsions. Whole blood, plasma, RBC, lung and diaphragm cholinesterase in IP dosed mice were inhibited as compared to controls.

Time of dosing and results of cholinesterase assays in the steer are presented in Table 7. No clinical signs or depression of whole blood, plasma or RBC cholinesterase were observed after intraruminal administration of algae. Rapid inhibition of whole blood, plasma and RBC cholinesterase, and clinical signs of salivation, tremors, dyspnea, recumbency and cyanosis were observed after parenteral administration of the algae extract. The steer died 75 minutes after the initial injection. No miosis or seizures were observed. Carotid and jugular blood pressure recordings did not reveal remarkable changes until the steer was recumbent and moribund. Postmortem examination revealed that the first IP injection had gone at least partially retroperitoneal.

Tissue cholinesterase determinations revealed normal brain cholinesterase activity, but depressed lung and diaphragm cholinesterase activities.

The nontreated algae-dosed pig developed clinical signs within 30 minutes. Thick salivation and mucoid nasal discharge comprised the initial clinical signs followed by bruxism, muscle tremors, muscle fasciculations, intermittent vomiting, defecation, urination, poor dorsal nasal capillary refill time, cold ears, dyspnea, cyanosis, mild struggling and death at 1 hr and 22 min after dosing. No miosis was observed. Arterial blood pO_2 and blood pH decreased and whole blood, plasma and RBC cholinesterase activities were inhibited (Table 8). Postmortem lung and diaphragm cholinesterase activity were inhibited compared to controls, but brain cholinesterase activity was not (Table 9). Postmortem examination revealed a 1.5 x 1.5 cm bulla in the dorsal diaphragmatic surface of the lung.

The control pig developed no clinical signs after dosing and was killed and necropsied at 1.5 hours postdosing. The treated algae dosed pig had experienced marked salivation at 19 minutes postdosing. Atropine and activated charcoal were administered at this time. Thereafter, salivation immediately ceased, but pronounced muscle fasciculations developed and persisted. Atropine was administered 2 more times over the next 12 hours when salivation returned. The pig never became dyspneic. Blood arterial pO_2 never dropped below 70 and blood pH never went below 7.40, therefore, there appeared to be no significant respiratory compromise in this pig. Whole blood, plasma and RBC cholinesterase activity progressively decreased after dosing and reached less than 10% of predose values within 1 hour (Table 10). Whole blood, plasma and RBC cholinesterase activities were still markedly depressed at 72 hours when the pig was killed. Postmortem brain, lung, and diaphragm cholinesterase

determinations, when compared to the control pig, suggested lung and diaphragm cholinesterase inhibition, but not brain cholinesterase inhibition (Table 9). Postmortem examination revealed charcoal to be still present in the spiral colon and colon.

Discussion

The blue-green algae identified in the case was Anabaena flos-aquae. Strains of this organism have been associated with previous field toxicoses and have been used in culture work to produce antx-a(s). High pressure liquid chromatography revealed a compound with the same retention time as antx-a(s). In addition, the evaluation of animals naturally exposed or dosed with either the algal cells toxin from the cells, and in vitro cholinesterase assays, revealed findings compatible with the effects of the cholinesterase inhibitor antx-a(s). Since the structure of antx-a(s) has not been determined, the toxin in this case cannot be said to be identical to the antx-a(s) toxin produced in the laboratory by the Anabaena flos-aquae clone NRC-525-17.

Profuse salivation and urination/defecation appeared to cause the ducks to seek out and drink considerable volumes of water after dosing. In nature this would probably result in cycles of further consumption of water containing toxin with increasingly severe toxicosis resulting in death. However, drinking of non-contaminated water might result in less severe toxicosis by dilution of toxin, or aiding removal of toxin through regurgitation or defecation. Death of birds after multiple doses would suggest that toxicosis can be cumulative on a daily basis. This study confirmed that antx-a(s) toxicosis occurs in avian species, and is consistent with most evidence suggesting that most field cases of antx-a(s) involve water fowl.

The pig appeared to be the most sensitive of the animals dosed intragastrically in this study with concentrated algae containing antx-a(s). Animals appeared to die from anoxia and acidosis secondary to cholinesterase inhibition and respiratory paralysis. Atropine and activated charcoal appeared to be lifesaving in one pig administered a lethal dose of toxic algae. In this pig, whole blood, plasma and RBC cholinesterase activity were severely inhibited to levels observed with death in the non-treated pig.

Anatoxin-a(s) appeared to be acting as a peripheral cholinesterase inhibitor due to lack of cental nervous system signs and lack of remarkable brain cholinesterase depression in algal dosed animals.

Cases of antx-a(s) toxicosis in wildlife and other domestic animals could be more common than previously believed due to rapid death of animals, the absence of methods to detect the toxin, and the apparent need to confirm peripheral cholinesterase depression in the tissues of the affected animals.

Table 1. Clinical signs observed in ducks after successive daily crop intubation with algae bloom material containing anatoxin-a(s).

Thick and profuse salivation
Regurgitation of algae
Diarrhea/urination
Muscle tremors
Depression
"Squinting" of eyes
Ataxia
Polydipsia
Vascular dilation of vessels in webbed feet
Dyspnea with open mouth breathing
Recumbency
Wing and leg paresis
Opisthotonus
Seizures (intermittent-tonic) just prior to death

Table 2. Plasma, brain, lung and breast muscle cholinesterase activities in ducks after successive intracrop administration of anatoxin-a(s) and brain cholinesterase activity in dead or clinically affected ducks from the field

Cholinesterase					
Plasma (uM/l/min)		Tissues (uM/g/min)			Status
Predose	Prior to Death	Brain	Lung	Breast Muscle	
1. 0.22	0.025	12.70	0.48	0.102	Terminated (moderate signs)
2. 0.27	<0.025	12.75	0.20	0.09	Died (2nd dose)
3. 0.27	0.26	12.73	0.78	0.27	Control
4. 0.29	0.27	12.92	0.78	0.36	Control
5. 0.26	<0.025	12.08	0.25	0.09	Died (5th dose)
6. 0.29	0.05	12.98	0.37	0.115	Terminated (mild signs)
7. 0.23	<0.025	13.00	0.14	0.08	Died** (4th dose)
8. —	—	12.70	—	—	Field death**
9. —	—	12.57	—	—	Field case (moderate signs)*

*moderate signs present at submission and when killed
**duck from affected farm
— = not determined

Table 3

In vitro inhibition of electric eel acetylcholinesterase by HPLC purified anatoxin-a(s) from the Tolono, Illinois field case.

Treatment[a]	A_{412}/min[b]	Percent Activity
Control[c]	0.434 (0.006)	100
2 x 10^{-4} ug/ul	0.242 (0.07)	56
4 x 10^{-4} ug/ul	0.184 (0.07)	42
1 x 10^{-3} ug/ul	0.156 (0.09)	36
4 x 10^{-3} ug/ul	0.000 (0.000)	0
1 x 10^{-2} ug/ul	0.000 (0.000)[d]	0

[a] enzyme and toxin are incubated for one minute before assay
[b] values reported as Mean (SEM)
[c] control contains enzyme, substrate, indicator but no toxin
[d] average of three experiments, all others are average of four experiments

Table 4. Whole blood, plasma, RBC, brain, lung and diaphragm cholinesterase activities in mice dosed orally (22 ml/kg body weight) with concentrated blue-green algae material containing anatoxin-a(s) produced by *Anabaena flos-aquae* from a pond in Tolono, Illinois

		Cholinesterase					
		(uM/l/min)			(uM/g/min)		
Mouse #	Dose	Whole blood	RBC	Plasma	Brain	Lung	Diaphragm
1	22 ml algae/kg BW	1.36	2.94	1.98	11.5	0.80	1.05
2	"	1.18	2.21	1.30	12.2	0.89	0.89
3	"	1.23	2.21	1.25	12.3	0.97	0.98
4	22 ml water (control)/ kg BW	1.14	2.64	1.20	13.7	1.00	1.15
5	"	1.17	2.20	1.78	14.8	1.06	0.85

Table 5. Tissue cholinesterase, and liver weights as a percent of body weight in mice (36.0 ± 1 gram) injected intraperitoneally with an extract of blue-green algae pond water containing antx-a(s) submitted to the University of Illinois Diagnostic Lab

Mouse no.	Dose	Time Surviving (minutes)	Liver % Bodyweight	Tissue Cholinesterase (uM/g/min) Brain	Lung	Diaphragm
Control	1.0 ml H_2O	killed (75 min)	0.051	12.73	0.80	1.86
1	1.0 ml Extract	5 min.	0.063	15.19	0.14	0.18
2	0.5 ml Extract	6 min.	0.059	15.02	0.25	0.55
3	0.25 ml Extract	14.5 min.	0.070	11.58	0.08	0.28
Average Values for Mice Dosed with Toxin			0.064	14.31	0.16	0.34

Table 6. Whole blood and plasma cholinesterase in mice (26 ± 2.5 gm) dosed intraperitoneally with an extract of blue-green algae pond water containing anatoxin-a(s) and decapitated just prior to death (10-15 minutes after dosing)

Mouse No.	Dose	Kidney % Bodyweight	Liver % Bodyweight	Cholinesterase (uM/l/min) Whole Blood	Plasma
Control	0.2 ml H_2O	0.018	0.052	1.75	2.54
1	0.2 ml Extract	0.02	0.056	<0.025	0.057
2	0.125 ml Extract	0.017	0.058	<0.025	0.036
3	0.05 ml Extract	0.018	0.059	<0.025	0.11
Mean Values for Mice Dosed with Extract		0.018	0.057	<0.025	0.07

Table 7. Cholinesterase activities of whole blood, plasma, RBC, brain, lung and diaphragm and blood gases in a steer dosed intraruminally and parenterally with algae bloom material containing antx-a(s).

INTRARUMINAL DOSING

Time	Cholinesterase (m/M/l/min) Whole Blood	Plasma	RBC
Predose	1.58	0.12	2.83
Dose 1			
1 hour	1.48	0.11	2.92
2 hours	1.52	0.098	2.92
24 hours	1.60	0.12	2.83
Dose 2 (24 hours)			
26 hours	1.62	0.14	2.67
36 hours	1.66	0.15	2.80
48 hours	1.82	0.14	2.83
72 hours	1.69	0.12	2.83

PARENTERAL ADMINISTRATION (RETROPERITONEAL/INTRAPERITONEAL)

	Cholinesterase (uM/l/min)			Blood Gases (mmHg)		Blood pH
Time	Whole Blood	Plasma	RBC	PO_2	PCO_2	
Predose	1.69	0.123	2.83	87.8	40.9	7.40
0 (dosed 300 ml supernatant)						
5 min	0.93	0.07	2.83	---	---	---
10 min	0.63	0.025	0.95	87.6	36.2	7.40
20 min	0.17	<0.025	0.16	99.3	39.6	7.39
30 min	0.13	<0.025	0.04	94.6	36.8	7.39
35 min (redose 250 ml supernatant)						
40 min	---	---	---	81.9	36.8	7.40
50 min	<0.025	<0.025	0.04	---	---	---
60 min	<0.025	<0.025	0.025	44.3	32.3	7.22
70 min	<0.025	<0.025	<0.025	41.0	46.5	7.32
75 min	---	---	---	41.0	52.6	7.26

--- = Not measured

Postmortem Tissue Cholinesterase (uM/g/min): Brain 4.48, Lung < 0.025, Diaphragm 0.07

Table 8. Whole blood, plasma, and RBC cholinesterase, blood gas and blood pH determinations in a pig (test A) given a lethal intragastric dose of blue-green algae cells containing anatoxin-a(s) and a pig (control) given an intragastric dose of distilled water.

Table 8a

Arterial Blood Gas* and pH Values

Time (min)	PCO_2		PO_2		Blood pH	
	Test	Control	Test	Control	Test	Control
Predose	41.8	37.4	85.2	100	7.40	7.45
30	37.2	39.8	84.3	100	7.38	7.43
60	33.4	39.0	86.8	100	7.41	7.43
75	37.2	37.4	—	91.7	7.31	7.42
82		—	51.6	—	—	—
90		35.3	—	100		7.46

*Units are in mmHg
— = Not analyzed

Table 8b

Cholinesterase Activities (uM/l/min)

Time (min)	Whole Blood		Plasma		RBC	
	Test	Control	Test	Control	Test	Control
Predose	1.39	1.50	0.27	0.26	2.20	2.34
30	0.07	1.37	0.06	0.23	0.07	2.47
60	<0.025	1.25	0.05	0.20	0.06	2.57
75	—	—	—	—	—	—
82	<0.025	—	0.04	—	<0.025	—
90		1.37		0.26		2.57

— = not analyzed
+ Detection limit

Table 9. Brain, lung, and diaphragm cholinesterase activities from: 1) a pig (A) administered a lethal intragastric dose of blue-green algae cells containing antx-a(s), 2) a pig (B) administered a suspected lethal dose of blue-green algae containing antx-a(s) and then treated with activated charcoal and atropine (pig survived), and 3) a control pig dosed intragastrically with water.

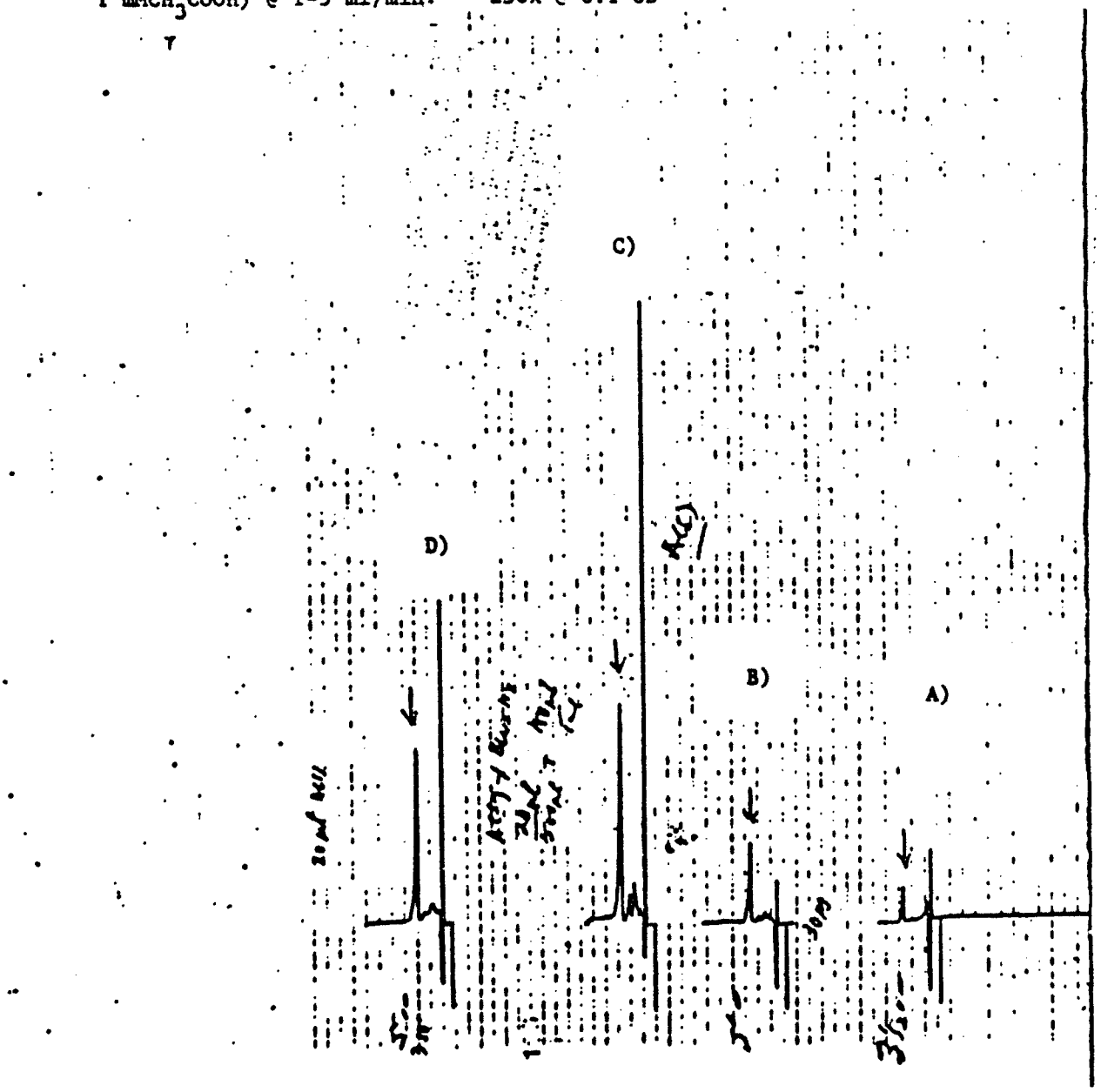
	Tissue Cholinesterase (uMol/g/min)		
Pig	Brain	Lung	Diaphragm
1. Lethal Dosed	5.60	0.09	0.09
2. Lethal Dosed and Treated with Activated Charcoal and Atropine	5.98	0.13	0.30
3. Control	6.02	0.27	0.45

Table 10. Whole blood, plasma, RBC, brain, and diaphragm cholinesterase determinations in a pig (C) given a potentially lethal dose of blue-green algae material containing antx-a(s) and then treated with activated charcoal and atropine.

Time	Cholinesterase (uM/l/min) Whole Blood	Plasma	RBC
Predose	1.10	0.32	1.80
5 min	0.92	0.25	1.16
10 min	0.64	0.11	1.03
19 min (atropine + charcoal)			
20 min	0.73	0.08	0.56
30 min	0.14	0.05	0.36
45 min	0.09	0.036	0.15
1 hr	<0.025	<0.025	0.11
1.5 hr	<0.025	<0.025	0.11
2.0 hr	<0.025	<0.025	0.05
3.0 hr	<0.025	<0.025	0.05
4.0 hr	<0.025	<0.025	0.11
4.5 hr (atropine)			
5.0 hr	<0.025	<0.025	0.05
7.0 hr	<0.025	<0.025	0.04
10.5 hr (atropine)			
12 hr	<0.025	0.07	0.05
24 hr	<0.025	0.07	0.04
48 hr	0.014	0.07	0.06
72 hr	0.18	0.07	0.09

Figure 1: High pressure liquid chromatograph spectrums of anatoxin-a(s) purified from Tolono, Illinois field case compared to anatoxin-c(s) standard (produced by Anabaena flos-aquae NRC-525-17).

Analytical HPLC Altex CN (4.9 mm x .15 cm) 80/20 (10 mM NH_4CH_3COO/ 1 mMCH_3COOH) @ 1-5 ml/min. 230λ @ 0.1 OD



A) Tolono toxin at 10 ug injection.
B) Tolono toxin at 30 ug injection.
C) Anatoxin-a(s) at 100 ug injection.
D) A(S) + Tolono toxin (35 + 30 ug) injection.

REFERENCES

1. Carmichael WW, Jones CL, Mahmood NA, et al.: Algal Toxins and Water-Based Diseases. CRC Critical Reviews in Environmental Control 1985:15, 275-313.

2. Carmichael WW, Mahmood NA: Toxins from Freshwater Cyanobacteria. Seafood Toxins, E. Ragelis Ed., 1984 ACS Symposium Series 262, ACS Publications, Washington, D.C.

3. Carmichael WW, and Gorham PR: Anatoxins from clones of Anabaena flos-aquae isolated form lakes of Western Canada. Mitt Internat Verein Limnol 1978:21.

4. Carmichael WW, Gorham PR: Anatoxins from clones of Anabaena flos-aquae isolated from lakes of Western Canada. Mitt Int Verein theor argew Jirmnol. 1978:21, 285.

5. Carmichael WW, Biggs D, Gorham P. Toxicological and pharmacologicl action of Anabaena flos-aquae toxin. Science. 1975:187, 542.

6. Carmichael WW, Biggs D, Peterson M: Pharmacology of anatoxin-a produced by the freshwater cyanophyte Anabaena flos-aquae NRC. Toxicon. 1979:17, 229-236.

7. Mahmood NA, Carmichael WW: The Pharmacology of Anatoxin-a(s), A Neurotoxin Produced By The Freshwater Cyanobacterium Anabaena Flos-Aquae NRC 525-17 Toxicon 1986:24,425-434.

8. Schwimmer M, and Schwimmer, D: Medical Aspects of Phycology. In: Algae, Man and Environment, (Jackson, DF, Ed.). New York: Plenum Press 1967, 279.

9. Skulberg OM, Codd GA and Carmichael WW: Toxic blue-green algal blooms in Europe: a growing problem. Ambio 13(4), 244.

10. Spiwak CE, Witkop B; Albuquerque Ex: Anatoxin-a: a novel, potent agonist at the nicotinic receptor. Mol Pharmacol 1980:18, 384-394

b. Anatoxin-a(s) field toxicosis in swine (Griggsville)

Four sows, one boar, and eight 18-23 kg swine died over a period of one week around a pond in Griggsville, IL in association with a blue-green algae bloom. Sudden death was the predominant clinical sign, but salivation, dyspnea, diarrhea, ataxia, open mouth breathing and recumbency were also observed. Gross postmortem examination of 4 pigs by the referring veterinarian revealed wet lung tissue.

Materials and Methods

Algae cells were collected in culture tubes and jars and shipped to Wright State University in cold-packs for light and scanning electron microscopic examination for algae identification, future culture, mouse bioassay and HPLC analysis. Brain, lung, and diaphragm were obtained from a sow that had died in conjunction with the field case. These were analyzed for chlorinesterase activity as described previously using the Modified Ellman Method. Mouse bioassay was also performed at UI using 3 Swiss Webster mice. Two mice were injected with 1.0 ml of bloom material, and 1 mouse with 1.0 ml of water for a control.

Results

Light microscopic and scanning electron microscopic examination of pond water containing algae by Nik Mahmood and researchers at Wright State University, Dayton, Ohio, revealed Anabaena flos-aquae, a blue-green algae, strains of which are known to be producers of antx-a(s). Mice injected with bloom material had clinical signs including thick salivation, mucoid lacrimation, dyspnea, cyanosis, clonic seizures and death. These clinical signs were compatible with toxicosis by antx-a(s). The mouse bioassay was repeated at Wright State University revealed similar results. Cholinesterase assay of brain tissue, lung and diaphragm from a sow that died on the farm revealed cholinesterase

activities of 4.42, 0.00, and 0.13 uM/g/min, respectively. Lung and diaphragm cholinesterase activities appeared inhibited, and the overall results were consistent with a peripheral acting cholinesterase inhibitor such as antx-a(s). Samples of algae in culture media and bloom material for freeze drying were sent to Wright State University for culture and toxin production, respectively.

7. SUMMARY

The start of formal studies with pigs may be temporarily delayed until the structure of antx-a(s) is known and a capability to absolutely assess toxin purity has been demonstrated. In the meantime, algae extracts of increasing purity can continue to be used to qualititively characterize pathophysiologic mechanisms of this toxin in mice and pigs, and its neurobehavioral effects on rats.

In the first year of study, the effects of antx-a(s) have been evaluated in mice, rats, swine, birds, and cattle. This enabled observation of the spectrum of clinical signs, that antx-a(s) produces, and insights into possible pathophysiologic mechanisms. Marked species differences were observed such as chromodacryorrhea in rats, profuse mucoid nasal discharge in pigs, seizures in rats, lack of toxicity after gavage in rodents and high toxicity with intragastric admistration in pigs. The actions of antx-a(s) are apparently due to acetycholine accumulation at the neuromuscular junction, pre- and postganglionic junctions in the autonomic parasympathetic system.

Possible pathophysiologic mechanisms of antx-a(s) were explored with electrocardiograph, blood pressure and electroencephalograph equipment, cholinesterase assays, gross postmortem and histopathologic evaluations. Information to date suggests that antx-a(s) causes death in animals from anoxia and acidosis secondary to respiratory paralysis

from cholinesterase inhibition. Preliminary blood pressure recordings in one pig indicated a marked rise in left pulmonary artery blood pressure, but further observation of this finding in other pigs is necessary before its significance can be determined. The majority of research data including brain cholinesterase determinations, neurological examinations and electroencephalograph recordings indicate that antx-a(s) does not cross the blood brain barrier and is not having a direct effect on the brain. Evidence for antx-a(s) having an effect on the brain comes from seizure activity, electroencephalographic alterations and narcosis observed in the rat. Further study with the rat is planned to clarify whether antx-a(s) is affecting the brain in this species.

The limited gastric or ruminal intubation studies using pigs, cattle, birds, mice and rats with antx-a(s) suggested that by this route the pig was the most sensitive species to toxicosis. The similarity in anatomy and physiology between the pig and man suggests that man could also be very susceptible to orally induced toxicosis. Intravenous and intramuscular atropine and gastric intubation of activated charcoal appeared to be a lifesaving treatment in one pig administered an intragastric lethal dose of algae containing antx-a(s).

Anatoxin-a(s) toxicosis was diagnosed in two field cases, one involving ducks and the other swine, in Illinois, in the first year. It was possible to harvest and freeze dry toxic algal material from blooms in these cases. Field cases of antx-a(s) and algal blooms producing antx-(s) may be more common than previously suspected due to the labile nature of the toxin and algae producing antx-a(s), lack of gross and histopathologic lesions, need for peripheral cholinesterase activity determinations, and due to the history of sudden death in animals. The

structure of antx-a(s) in these blooms can be compared in the future for structural variants to the laboratory-produced antx-a(s) from Anabaena flos-aquae NRC 525-17.

STUDIES ON CYANOBACTERIA (BLUE-GREEN ALGAE) NEUROTOXINS: I. PURIFICATION OF ANATOXIN-A(S) AND ANATOXIN-A II. KINETIC ANALYSIS OF CHOLINESTERASE INHIBITION BY ANATOXIN-A(S)

Nik A. Mahmood, PhD

CONTENTS

INTRODUCTION

Different strains of *Anabaena flos-aquae* have been shown to produce toxins (anatoxins) which are physiologically distinct.[1] Two of these anatoxins, anatoxin-a obtained from strain NRC 44-1 and anatoxin-a(s) from NRC 525-17 have been studied. The chemical structure and pharmacological action of anatoxin-a is already known. It is the alkaloid 2-acetyl-9-azabicyclo non-2-ene[2] and is a potent depolarizing neuromuscular blocking agent at both the nicotinic and muscarinic receptors.[3]

The term anatoxin-a(s) was derived from anatoxin-a since it was first thought that the two toxins shared similar pharmacological properties. But it was later found that their pharmacological actions differed [4,5] and most recently it was shown that anatoxin-a(s) acts as an irreversible anticholinesterase.[6,7] Measures to characterize the fundamental chemical properties of anatoxin-a(s) have shown it to be a positively charged compound of molecular weight > 500. It is acid stable, alkaline labile, water soluble, unstable in organic solvents and exhibits weak UV absorbence at 200-400 nm.

The present studies with the anatoxin-a(s) and anatoxin-a involve the development of large-scale (preparative) purification method: the aim is to produce larger quantities of toxin without reducing purity. The other part of the study involves the elucidation of the mechanism of action of anatoxin-a(s). This has primarily been directed toward the biochemical characterization of the inhibition of cholinesterase by anatoxin-a(s).

The cholinesterase inhibition studies are necessary for several reasons:

1. to support the earlier pharmacological and toxicological data that anatoxin-a(s) is an anticholinsterase.
2. to elucidate the mechanism of irreversible action of anatoxin-a(s).
3. to compare the inhibition between different sources of cholinesterase.

MATERIALS AND METHODS

a. Toxin extraction

1. Anatoxin-a(s)

The extraction and purification of anatoxin-a(s) from Anabaena flos-aquae NRC 525-17 are shown in the flow diagram (Fig. 1). The extraction procedure was developed as part of the author's dissertation work.[6] Since then, some modifications were made to accommodate the large-scale extraction and purification.

Lyophilized cells (20 g) in 250 ml acidified ethanol (pH 4.0 acidified with acetic acid) were stirred for 24 hr. The suspension was centrifuged at 8,000-10,000 rpm for 1 hr at 4°C. If necessary, the residue was re-extracted by repeating the above procedure until no further toxicity was observed. The supernatants were pooled and concentrated by air drying in a heated bath (40°C). The dried extract was dissolved in 20-50 ml 0.1 M acetic acid and loaded on Bond Elut (C_{18} Analytichem) cartridges. The cartridges were stacked two columns

high and a total of four or six cartridges were used. These cartridges had to be prepared before loading the extract with 2.0 ml methanol followed by 5.0 ml water. The extract was then loaded and the eluant collected. The cartridges were washed with 5 ml 0.1 M acetic acid and the eluant collected again. The pooled eluant was air-dried and reconstituted in 10-20 ml of 0.1 M acetic acid and centrifuged at 10,000 rpm at 4°C for 1 hr. If the supernatant appeared to be darkly colored, then the step using Bond Elut was repeated. Otherwise, the sample was ready for gel filtration.

2. Anatoxin-a

Anatoxin-a from Anabaena flos-aquae NRC 44-1 was extracted using the same method as for antx-a(s) as shown in the flow diagram (Fig. 1).

3. Column Chromatography

The following method was used for the separation of both anatoxin-a and anatoxin-a(s). Sephadex G-15 (40-120 u size, Sigma) was rinsed, swollen and deaerated in water overnight. The column (Pharmacia C-26 column, 2.6 x 70 cm) was slurry packed under gravity flow using a packing reservoir. The final column bed volume was 230 ml. The column was washed with 0.1 M acetic acid for at least 1 bed volume initially or following the previous run at a constant flow rate of 0.4 ml/min. The eluant was supplied by a peristaltic pump (Gilson Minipuls 2). The extract in 5.0 ml (0.1 M acetic acid) was applied to the column using a sample loader. The effluent, monitored at 230 nm was collected in fractions of approximately 6-7 ml volume using a Gilson fraction collector (Model FC-80k).

Pooled fractions, in which the presence of toxin was confirmed by mouse bioassay, were loaded on a Sep-pak C_{18} (Waters) cartridges for separating crude toxins from some pigments. The procedure for using

Sep-pak C_{18} cartridges was similar to the one described for Bond Elut (Analytichem) cartridges (above). The pooled eluants were then ready for HPLC.

b. High performance liquid chromatography (HPLC)

In the HPLC runs for both anatoxin-a(s) and anatoxin-a, Waters semi-preparative CN (cyano) columns (7.8 x 300 mm) were used. The conditions for the runs were as follows: 1) 100% water for 15 min; 2) for anatoxin-a(s), 0-6 mM ammonium acetate was used in a 10 min linear gradient; for anatoxin-a, a linear gradient from 0-8 mM ammonium acetate over 15 min was used; 3) for anatoxin-a(s), 10% 6 mM ammonium acetate was used in an isocratic run for 10 min and for anatoxin-a, 100% 8 mM for 15 min was used; 4) 100% water was then used in 5 min linear gradient.

The toxic peaks were pooled and rerun on a second CN Column (7.8 x 300 mm). Sometimes is was necessary to run specimens on the analytical column (Altex CN, 4.5 x 150 mm) with 10 mM ammonium acetate: 1 mM acetic acid (80:20) at 1.5 ml/min to separate side peaks and reduce sample discoloration. The flow rate for the semi-preparative run was 2 ml/min and the peaks were monitored for absorbence at 230 nm.

A preparative column, custom packed Delta 500 CN (5 x 29 cm) (Waters) was also tried to purify anatoxin-a(s) in a number of preliminary trial runs. The gradient elution is still in preparation.

c. Verification of anatoxin-a(s) purity

Two methods were used to check purity: 1) HPLC using the analytical column as described above and 2) thin layer chromatography (TLC). Three solvent systems were developed for use in addition to the one developed by Andrew Dahlem at University of Illinois (personal communication). The three solvent systems are listed below:

1. pyridine: ethyl acetate: acetic acid (15:5:3)

2. pyridine: ethyl acetate: acetic acid: water (15:5:3:4)

3. n-butanol: acetic acid: water (2:1:1)

The TLC plates (Silica Gel 60, E. Mark) were heated at 110°C for 30 min. The 10 ul spots (5-10 ug toxin) were applied and the plates developed in the respective solvent systems. The plates were air dried, inserted into an iodine tank for 10-15 min, heated at 110°C 10-15 min and observed under long wave UV.

d. Cholinesterase inhibition

A photometric method of Ellman et al[7] was used to determine the in vitro cholinesterase activity.

Acetylcholinesterase, (EC 3.1.1.7) from electric eel type V-S, and human erythrocyte type XIII and horse serum butyrylcholinesterase (EC 3.1.1.8) were obtained from Sigma. A stock solution of each enzyme in 0.1 M KH_2PO_4 buffer pH 8.0 was kept frozen. For each assay 0.25 U of enzyme were used. All assays were carried at room temperature.

For each assay, microliter volumes of anatoxin-a(s) were added to one side of the cuvette and 0.25 U enzyme to another corner, followed by addition of 3.0 ml phosphate buffer pH 8.0 to the cuvette. The solution was mixed and incubated for various times. After incubation, the substrate (acetylthiocholine or butyrylthiocholine at 0.075M) was added, and the change in absorbence at 412 nm recorded.

RESULTS

a. Purification

In the gel filtration run (Sephadex G-15), anatoxin-a(s) was found to be eluted after only 40-50% of the column bed volume had passed. The toxins were eluted together with the light yellow pigments. In the previous technique[6], the final step in the purification of anatoxin-a(s) employed the use of an

Altech CN and/or Zorbax CN (9.4 x 250 mm) which was run isocratically with 10 mM ammonium acetate and methanol (50:50). In anticipation of an increased scale of purification using the Waters Delta preparative HPLC 3000 system, it was necessary to determine whether a Waters CN semi-preparative column would work for the separation of anatoxin-a(s).

Good results were obtained with a modified technique that utilized a linear gradient of water to 6 mM ammonium acetate in 25 min. The separation profile of anatoxin-a(s) using a Waters CN column (7.8 x 300 mm) is shown in Fig. 2 with a retention time of 25 min. It was observed that the toxin eluted from a single run on the semi-preparative column contained side peak contaminants and discolored products as shown by the repeat run on the semi-preparative column (Fig. 3).

The discoloration of the collected semi-preparative run peaks were likely a result of pigment contamination. These pigments were apparently leached very slowly from the semi-preparative column. Depending on the appearance of the toxin collected from the second HPLC semi-preparative rerun, it is sometimes necessary to rerun the pooled toxins on an anlytical column. A typical analytical HPLC profile of anatoxin-a(s) is shown in Fig. 4. The purity of the toxic peak was further verified using TLC in all four solvents which gave a single spot with a minor spot at the origin that corresponded to the pigment. The average yield of toxin obtained from the 20 g extracted is about 1 mg.

Anatoxin-a was extracted using the same method as for anatoxin-a(s). In the gel filtration run (Sephadex G-15), anatoxin-a was found to be eluted after between 100-120% of the column bed volume had passed. In the HPLC run using the Waters CN (7.8 x 300 mm) semi-preparative column, anatoxin-a was eluted with a higher ammonium acetate concentration than anatoxin-a(s) (Fig. 5). This corresponds very well to the results with ion-exchange

chromatography using CM-Sephadex C-25 (weakly basic cation exchanger), where anatoxin-a was eluted with 1.0 M acetic acid and anatoxin-a(s) with 0.3-0.5 M acetic acid. The ion-exchange chromatography was used in the early stages of methods development but in the current method, ion-exchange has been eliminated.

As with the anatoxin-a(s), the toxin collected from the first HPLC run was slightly discolored. It was necessary to rerun the toxin on the second HPLC column in order to eliminate the pigments.

b. Cholinesterase inhibition

The inhibition of eel acetylcholinesterase (EC 3.1.1.7) by anatoxin-a(s) is shown in Fig. 6. The inactivation of eel acetylcholinesterase by anatoxin-a(s) was time and concentration-dependent. The inactivation followed first-order kinetics, indicative of reversible binding of the inhibitor, followed by irreversible inhibition. Likewise, the inhibition of human erythrocyte acetylcholinesterase (EC 3.1.1.7) by anatoxin-a(s) was time and concentration-dependent (Fig. 7). The pattern of inhibition resembled that of eel acetylcholinesterase; but further comparison indicated that the inhibition by anatoxin-a(s) was greater towards human erythrocyte acetylcholinesterase than eel acetylcholinesterase. This was done by comparing the percent inhibition at a fixed time versus the toxin concentrations used. The crude preparation of human erythrocyte acetylcholinesterase might have contributed to the observed differences. The inhibition of horse serum cholinesterase (or butyrylcholinesterase) (EC 3.1.1.8) by anatoxin-a(s) was found to be less specific than the inhibition of acetylcholinesterase. The inhibition of the butyrylcholinesterase. The inhibition of the butyrylcholinesterase was observed to be non-linear (Fig. 8). The nonlinearity was shown to be biphasic when the inhibition was carried out a lower concentration of anatoxin-a(s).

DISCUSSION

One of the major problems with the large-scale purification of the neurotoxins is the presence of chlorophyll and other pigments which on many occasions were eluted with the purified toxin. A number of steps have been taken to reduce or eliminate contaminations from these pigments. In the earlier stage of extraction, this was achieved by the use of disposable preparative cartridges, either Bond Elut (Analytichem) or Sep-pak C_{18} (Waters). In order to avoid 'leached' pigments in the columns during HPLC runs, the pooled toxin after the first run was rerun (purified) on a second column. If there were side peaks present in the pooled repurified toxin, the toxin was further separated on an analytical HPLC column.

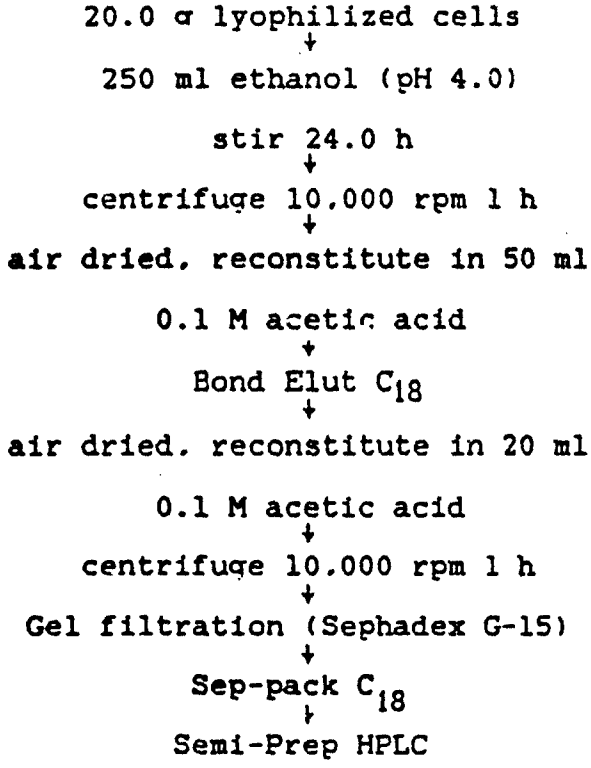
The present HPLC technique is time consuming. An attempt to scale-up the purification of anatoxin-a(s) to the preparative scale has been achieved in order to reduce running time. It was carried out on the custom made preparative cyano column (Delta 500, 50 x 290 mm) with the same mobile phase as that used for the semi-preparative runs.

The cholinesterase inhibition studies led to several conclusions. The irreversibility of the inhibition of anatoxin-a(s) on eel acetylcholinesterase has been shown using equilibrium dialysis and a plot of Vmax versus total enzyme.[6] It was also observed that diluting the inhibited enzyme up to 100 times with pH 8.0 phosphate buffer did not cause any reversibility.

The irreversible action of anatoxin-a(s) seemed to occur without first forming the reversible enzyme-anatoxin-a(s) complex as shown by the plot of first-order rate constants (p) against increasing anatoxin-a(s) concentrations (Fig. 9). This, if it is truly happening, is interesting because all known irreversible anticholinesterases (i.e. organophosphates) with the exception of VX [o-ethyl S-2 (diisopropylamino) ethyl methyl phosphonothioate][8] form a reversible complex with the enzyme prior to the irreversible inhibition. All

of these irreversible compunds act on the enzyme active/peripheral sites. This would make anatoxin-a(s) a non-specific inhibitor of acetylcholinesterase. A number of experiments can be done to show this, i.e. the pH effect, the substrate and the presence of reversible inhibitor on the activity of anatoxin-a(s).

The nonlinear character of the inhibition of butyrylcholinesterase by anatoxin-a(s) could be due to the presence of two or more different forms of the enzymes which are reacting at different rates from one another. These different forms could be either different subsites on the same enzyme, which react at different rates with anatoxin-a(s), or a mixture of different isozymes.



*repeat Bond Elut Step if supernatant is darker in color

Fig. 1. Flow diagram for the isolation of anatoxins-a(s) and anatoxin-a

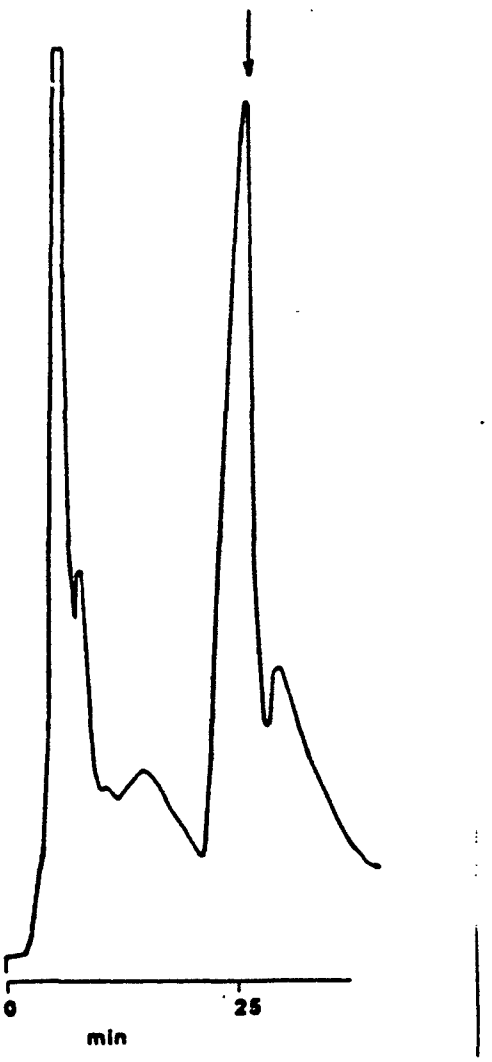


Fig. 2 Semi-preparative HPLC of anatoxin-a(s) (↓).

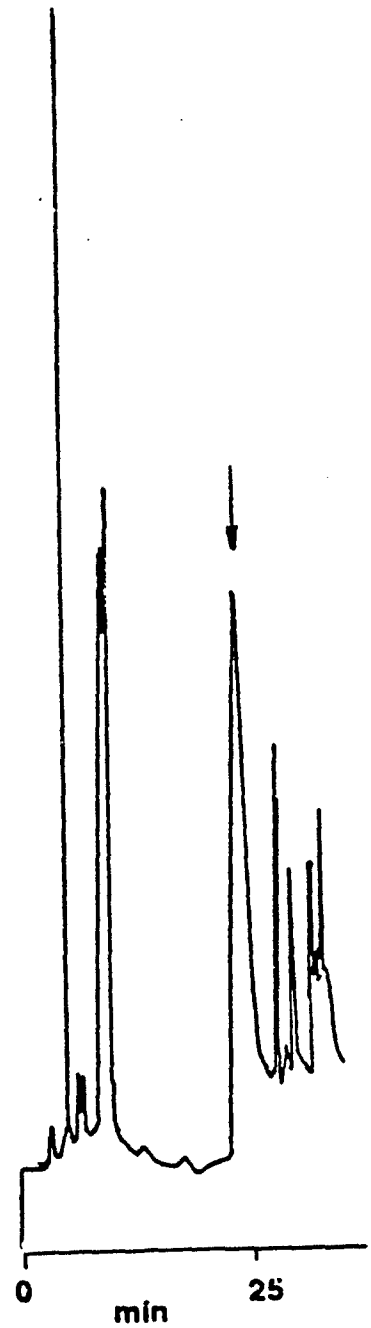


Fig. 3 Semi-preparative HPLC of anatoxin-a(s) (↓).

Fig. 4 HPLC (analytical) separation of anatoxin-a(s) (+) Column - Altex CN (4.6 x 150 mm).

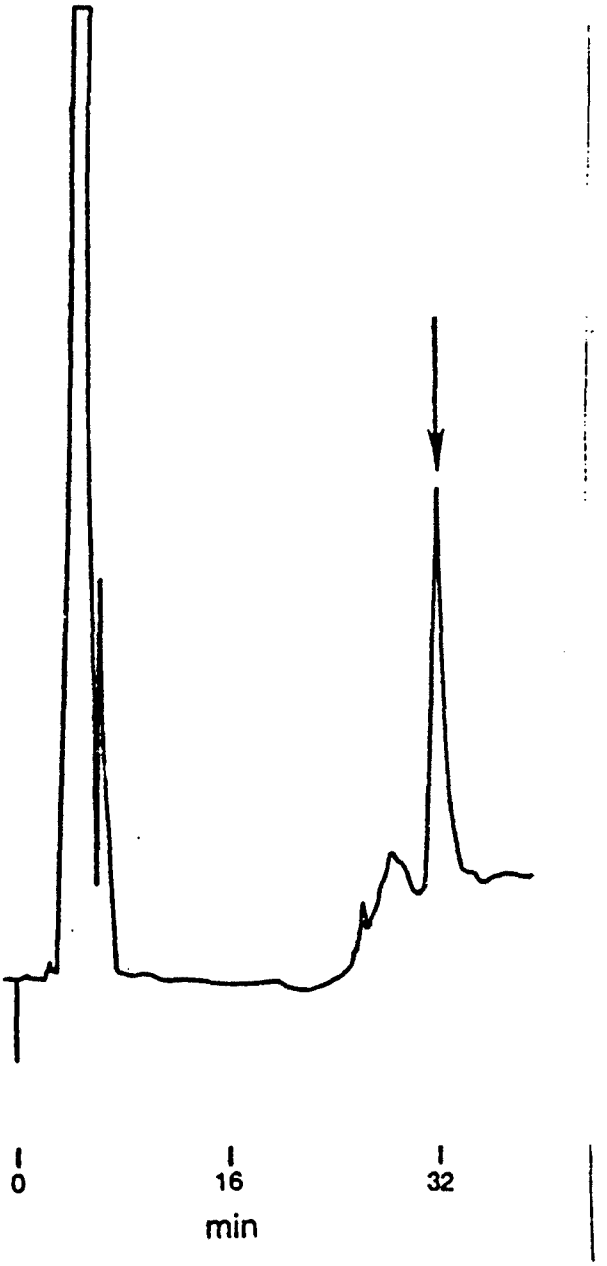


Fig. 5 HPLC separation of antx-a (↓) on Waters CN column (7.8 x 300 mm).

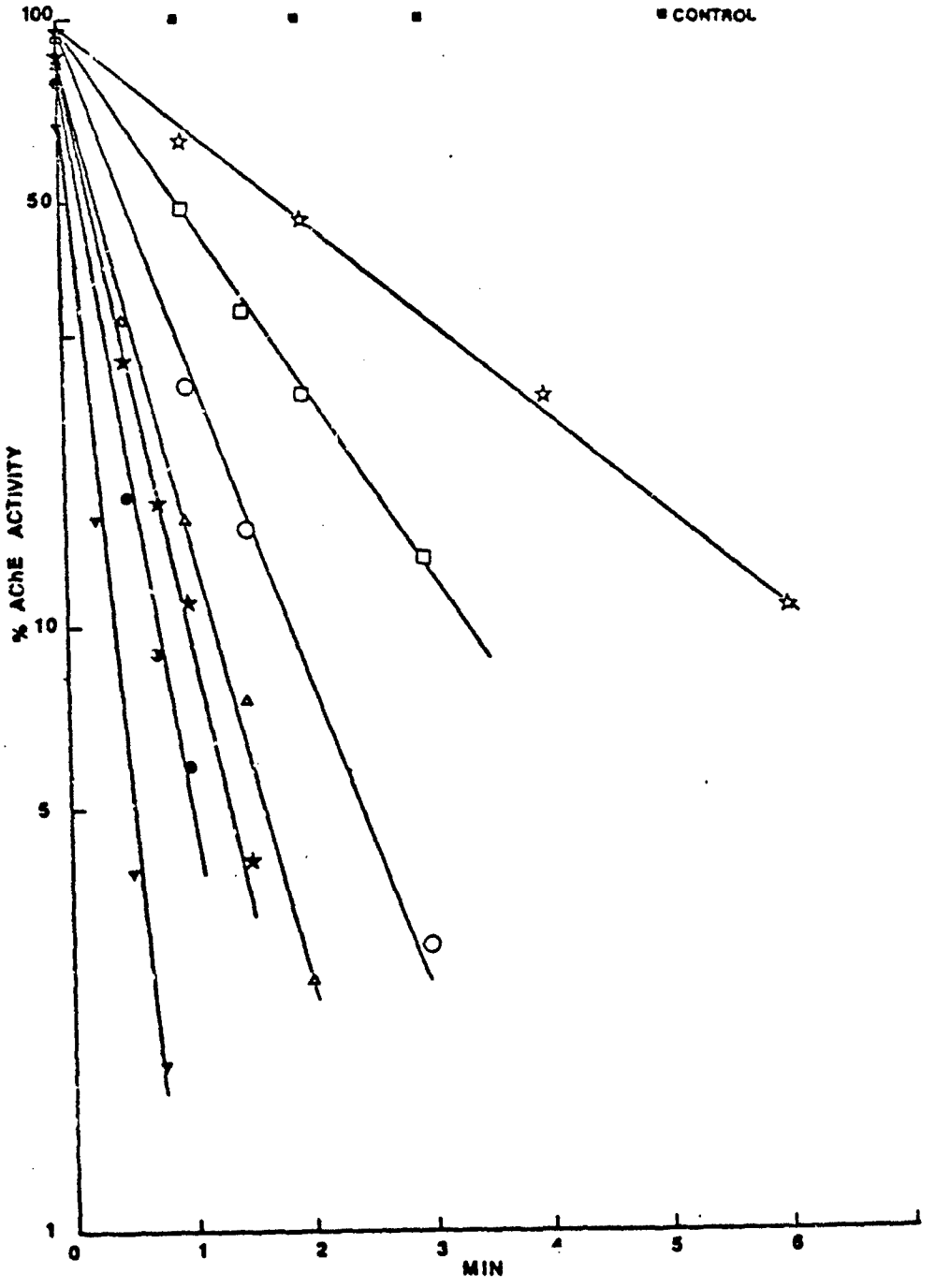


Fig. 6 Progressive irreversible inhibition of eel acetylcholinesterase by antx-a(s).

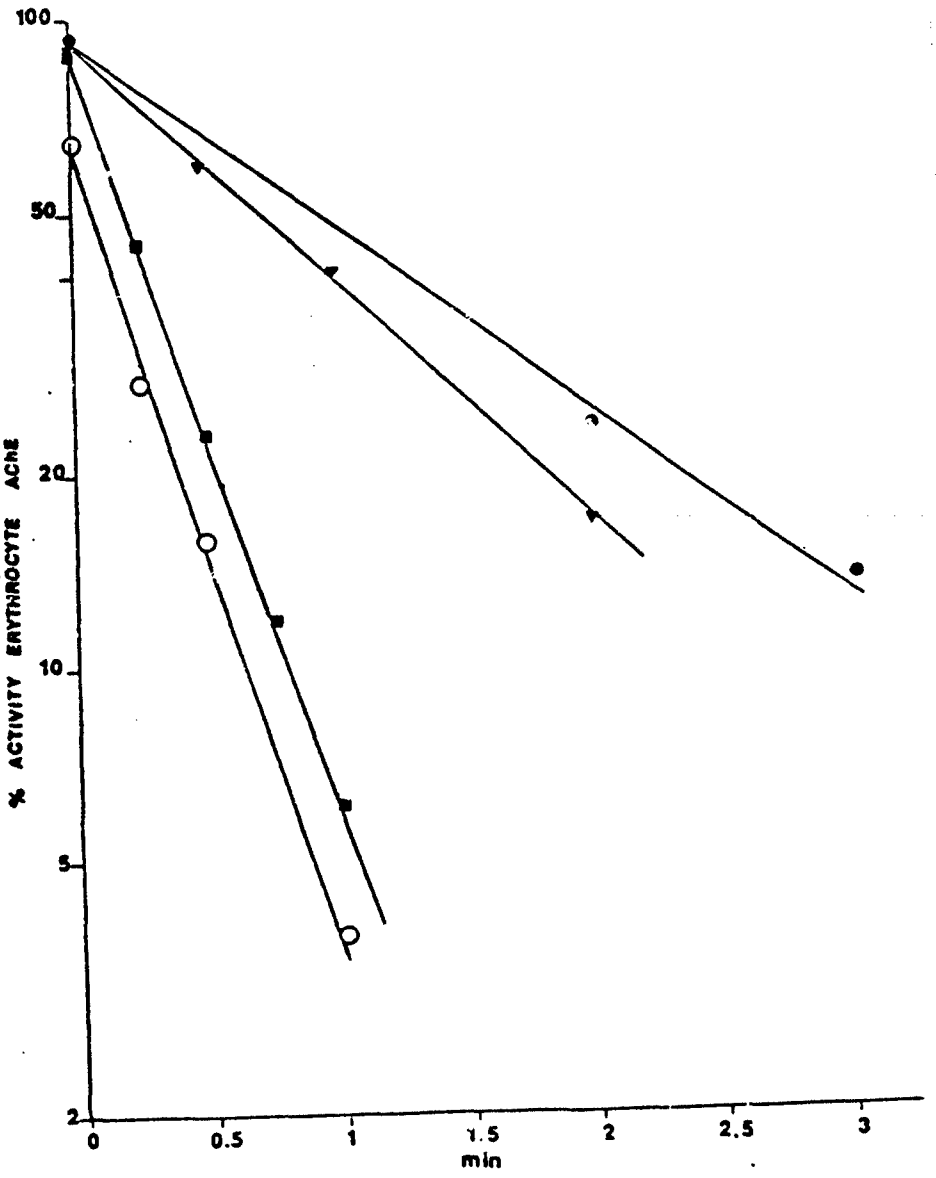


Fig. 7 Progressive irreversible inhibition of human erythrocyte acetylcholinesterase by antx-a(s).

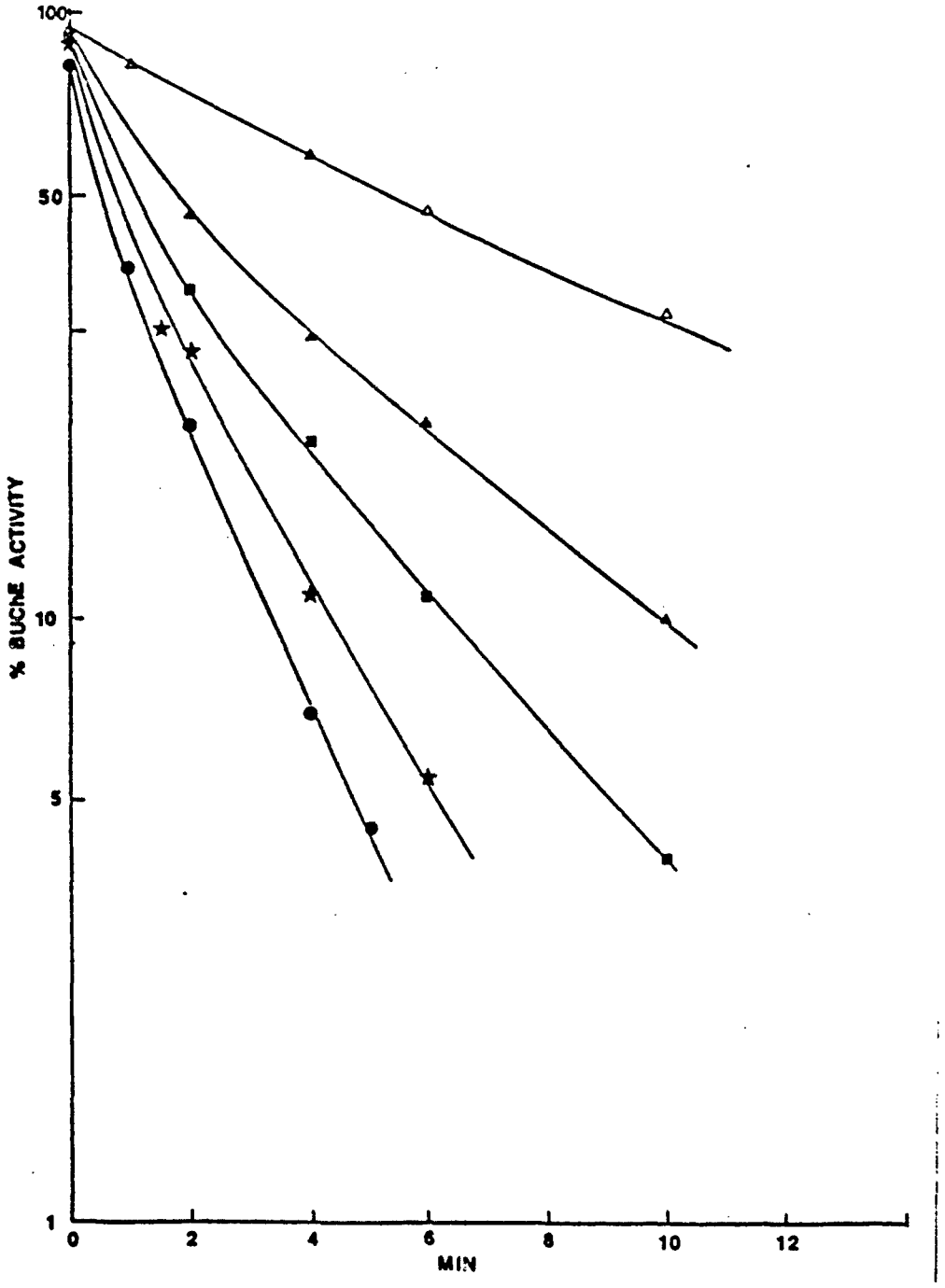


Fig. 8 Progressive irreversible inhibition of horse serum cholinesterase by antx-a(s).

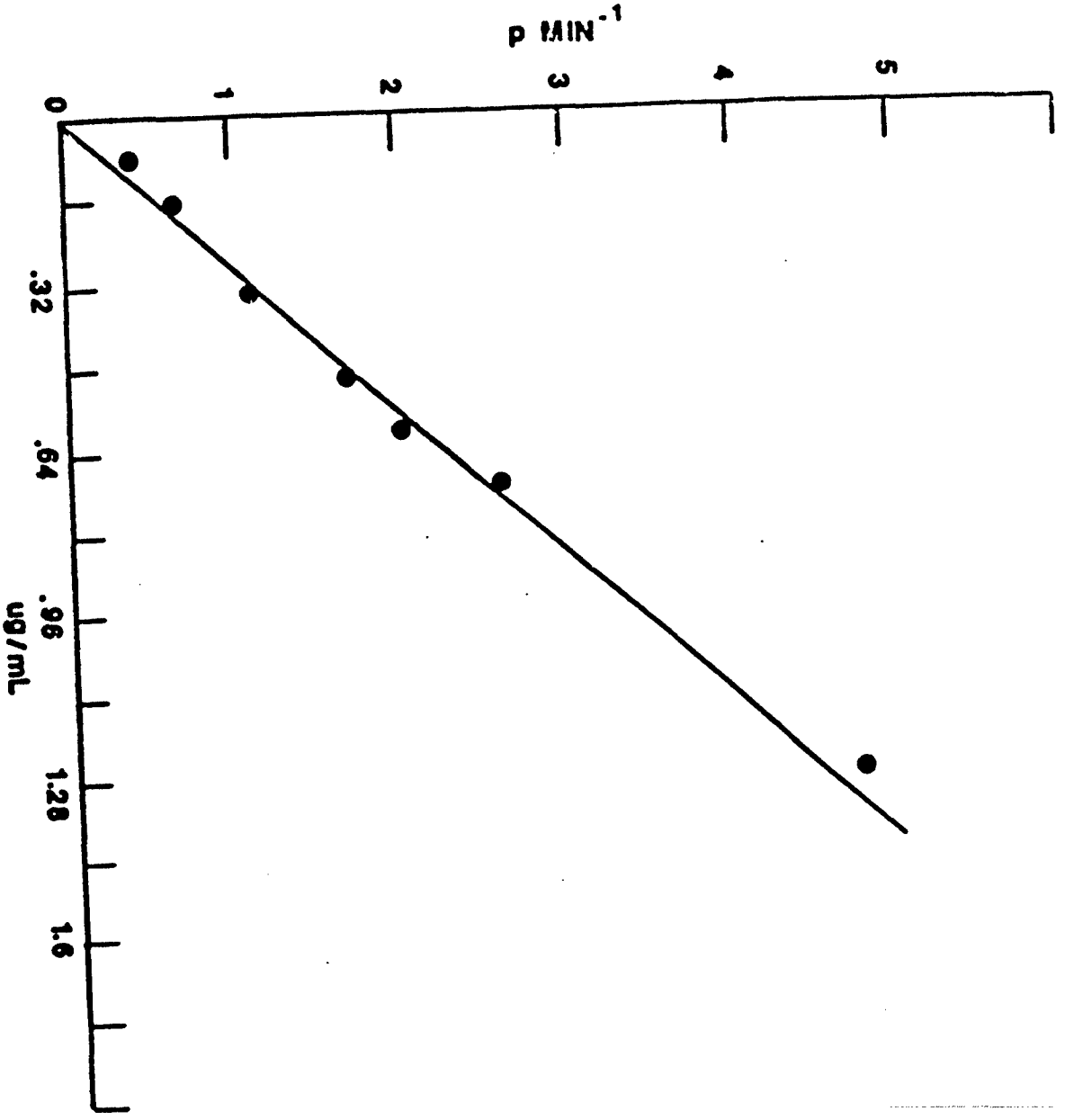


Fig. 9 Plot of first-order rate constants versus antx-a(s).

FIGURE LEGENDS

Fig. 2 Semi-preparative HPLC of anatoxin-a(s) (↓). Column - Waters CN (7.8 x 300 mm). Conditions: mobile phase - water to 6 mM ammonium acetate. 0-15 min of 100% water and linear gradient in 10 min. flow rate - 2 ml/min: defection - 230 nm @ 0.2 AUFS. Sample injected from pooled G-15 gel filtration.

Fig. 3 Semi-preparative HPLC of anatoxin-a(s) (↓). Same conditions as in Fig. 2, but the injected sample is the pooled toxic peak obtained from the first run.

Fig. 4 HPLC (analytical) separation of anatoxin-a(s) (↓) Column - Altex CN (4.6 x 150 mm). Conditions: 10 mM ammonium acetate: 1 mM acetic acid (80:20), 1.5 ml/min., 230 nm. 0.2 AUFS.

Fig. 5 HPLC separation of antx-a (↓) on Waters CN column (7.8 x 300 mm). Conditions: 100% water (15 min.) --> 0 to 8 mM ammonium acetate. linear gradient in 15 min --> 100% 8 mM ammonium acetate (15 min) at 2 ml/min. 230 nm. Antx-a peak (↓).

Fig. 6 Progressive irreversible inhibition of eel acetylcholinesterase by antx-a(s). Antx-a(s) concentrations, ug/ml: (☆) - 0.083; (□) - 0.166; (○) - 0.331; (△) - 0.497; (✱) - 0.599; (●) - 0.662; (▼) - 1.262. Each point represents the mean of 3 of 4 determinations.

Fig. 7 Progressive irreversible inhibition of human erythrocyte acetylcholinesterase by antx-a(s). Antx-a(s). Antx-a(s) concentrations. ug./ml: (●) - 0.16; (▼) - 0.32; (■) - 0.66; (○) - 1.26. Each point represents the mean of 3 determinations.

Fig. 8 Progressive irreversible inhibition of horse serum cholinesterase by antx-a(s). Antx-a(s) concentrations. ug/ml: (△) - 0.331; (▲) - 0.662; (■) - 0.994; (★) - 1.325; (●) - 1.656. Each point represents the mean of 3 or 4 determinations.

Fig. 9 Plot of first-order rate constants versus antx-a(s).

REFERENCES

1. Gorham, P.R., Carmichael, W.W. (1979) Pure and Applied Chem. 52, 165.

2. Huber, C.S. (1972) Acta crystallogr. B28, 2577.

3. Carmichael, W.W., Biggs, D.F. and Peterson, M.A. (1979) Toxicon 17(3), 229.

4. Carmichael, W.W. (1982) S. Afr. J. Sci. 18, 367.

5. Mahmood, N.A. and Carmichael, W.W. (1986) Toxicon. 24(5) 425.

6. Mahmood, N.A. (1985) Ph.D. Thesis, Wright State Univ., Dayton, Ohio 45435, 166 p.

7. Mahmood, N.A. and Carmichael, W.W. (1986) Abstract presented at 2nd American Symposium on Animal, Plant and Microbial Toxins, May 21-23 in Scottsdale, Arizona.

8. Ellman, G.L., Courtney, K.D., Andres, Jr., and Featherstone, R.M. (1961) Biochem. Pharmac., 7, 88.

9. Radic, Z., Reiner, E., and Simeon, V. (1984). Biochem. Pharmac. 33(4), 671.

Annual Progress Report for the Period September 1, 1985 to August 31, 1986

PRELIMINARY STUDIES WITH ANATOXIN-A

Drs. James Fikes and Lloyd Barr

CONTENTS

INTRODUCTION

Investigation of the cyanobacterial neurotoxin anatoxin-a, produced by Anabaena flos-aquae, was begun in the fourth quarter of this contract year. One goal of this study was the establishment of a dose-response relationship for anatoxin-a in rats. Previous studies have involved characterization of various pathophysiologic effects of anatoxin-a in isolated systems. In general, however, data, on the ability of the toxin to exert specific life threatening effects when administered to intact animals, has not been generated. A second goal, therefore, was the development of a method to stimulate the phrenic nerves of anatoxin-a dosed animals to enable determination of whether phrenic nerve-diaphragmatic neuromuscular transmission is blocked by the toxin.

MATERIALS AND METHODS

a. Toxin

"Semipurified" anatoxin-a (antx-a) extract was used in all studies thus far since purified toxin is as yet unavailable. The "semipurified" toxin was extracted (see Figure 1) by adding lyophilized cells to ethanol (pH 4.0, adjusted with acetic acid) and stirred for 18 hours. The solution was then centrifuged and the supernatant fluid was removed and filtered. The filtrate was then passed through a preparative cartridge, Bond Elut (Analytic Chem). The final filtrate was then dried down and reconstituted with saline to a concentration of 0.1 g dry cell material/ml of extract equivalence and frozen. The "semipurified" antx-a at this point is a clear green solution. Toxicity was verified by mouse bioassay. Purity of the solution was not determined due to the unavailability of a pure toxin standard. All doses of "semipurified" antx-a used in this phase of the study came from the same batch of extracted cell material.

b. Animals

Male, Harlan Sprague Dawley SD rats weighing between 283 and 375 grams were injected with varying doses of the "semipurified" antx-a intravenously (IV) as well as intraperitoneally (IP) for comparison.

c. Intravenously Dosed Rats

Five rats were injected IV with antx-a via a catheter in the tail vein. Doses are shown in Table 1. All 5 animals in the IV dosing group were anesthetized with methoxyflurane and weighed. A 26 gauge Angiocath^{R} was then placed in one of the tail veins. The rats were allowed 45 to 75 minutes to recover from the anesthetic prior to dosing with the "semipurified" toxin.

d. Intraperitoneally Dosed Rats

For comparison effects after IV administration, 5 rats were injected IP with varying amounts of "semipurified" antx-a with two of the rats being injected twice. The second injections were given after a recovery period of 1 week.

e. Necropsy Procedure

Animals which died following treatment were immediately necropsied. The skin, brain, skeletal muscles, thoracic and abdominal organs were examined grossly. The lungs were fixed via intratracheal instillation of 10% neutral, buffered formalin. Sections of tissues, including liver, lung, small intestine, kidney, spleen, myocardium, spinal cord and brain, were fixed by immersion in 10% neutral, buffered formalin. These tissues were routinely processed, embedded in paraffin, sectioned at 6 um, and stained with hematoxylin and eosin.

f. Preliminary Development of Techniques to Evaluate Phrenic Nerve Stimulation in Animals Given Antx-A

Development of a technique to electrically stimulate the phrenic nerve in the live-anesthetized animal was begun this quarter. Three rats were anesthetized with IP injections of pentobarbital and four rats by IV administration. A ventral midline incision was made beginning at the sternum and extending anteriorly approximately 2 1/2 cm along the ventral neck. The phrenic nerve was exposed by sharp and blunt dissection at the point it branches off the Vth cervical nerve and crosses the VIth and VIIth cervical nerves. Initially used spade electrodes were replaced by cuff electrodes.

RESULTS

a. Intravenously Dosed Rats

Survival data is shown in Table 1. Animals that survived were observed for 7 days postdosing. The two animals which survived the IV dosing (i.e. rats 1 and 4), showed clinical signs but appeared to recover fully based on clinical observations. Rat 1 was unable to walk immediately after the IV injection but could crawl. This appeared to be due to a moderate muscle weakness. When the rat did move, its head and limb motions were exaggerated. This animal could walk by 26 minutes postdosing and appeared clinically normal by 30 minutes postdosing. At no time did this rat appear unresponsive to external stimuli or exhibit signs of labored breathing. Rat 4 was also unable to walk immediately after dosing but was able to crawl. Immediately after injection, the rat's left hindlimb appeared paretic. Although the rat could move the leg, it was extremely weak as compared to the other legs and had no pedal reflex. At one minute after dosing, the rat had an increased respiratory rate with generalized muscle weakness. The rat had an intact righting reflex at all times. By 4 minutes postdosing, the left hindlimb paresis appeared to move anteriorly and partially affected the left forelimb. The rat did not respond to needle pricks on the left side either laterally or dorsally from the hindlimb to the shoulder. At 9 minutes postdosing, the rat began to respond to pinching of the toes of the left hindlimb and began steady improvement in the use of its left fore- and hindlimb. The rat improved quickly during the next 10 minutes. Improvement tapered off during the next 16 minutes but, by 35 minutes postdosing, the rat appeared clinically normal.

The three rats given lethal doses IV (see Table 1) demonstrated a fairly consistent progression of clinical signs. Rats 2 and 3 were

unable to walk by the time the animals were placed in the box for observation (approximately 5 seconds after the start of the injection). Rat 5 was able to walk for approximately 10 seconds after being placed in the box but then it too began demonstrating the generalized muscle weakness and quickly progressed to only being able to crawl. Each of the three rats was able to crawl for only 15-60 seconds. Clinical signs occurred fairly consistently in the order shown in Table 2.

b. Intraperitoneally Dosed Rats

Four of the rats injected intraperitoneally once and one injected twice survived (see Table 3). The rats which survived demonstrated a variety of clinical responses to IP dosing from no change to moderate unresponsiveness to external stimuli and inability to walk.

After the first dosing, rats 6 and 7 did not exhibit any clinical effects. Rat 9 began to experience labored breathing 12 minutes after dosing. By 18 minutes postdosing, the rat could barely walk and, by 20 minutes postdosing, it could only crawl. Respiration continued to be labored until 27 minutes after dosing when the dyspnea began to ease and crawling activity began to increase. By 56 minutes postdosing, respiration appeared normal. Although the rat could walk, its limb and head movements were exaggerated. Clinical recovery appeared complete by 6 1/2 hours.

Rat 10 appeared normal until 9 minutes postdosing. At that time, it began to walk with exaggerated motions of the limbs and had an increased respiratory rate. The respiratory rate and abnormal limb movement continued to increase for the next two minutes. This appeared to peak by 11 minutes postdosing, and then began to slowly improve. At 36 minutes postdosing, the rat was still somewhat unresponsive to stimulation and had slightly exaggerated limb movement but normal respiration. By eight hours postinjection, the animal appeared clinically normal.

Rat 6 was injected a second time IP, 7 days following the first injection. At six minutes postdosing, it exhibited a reduction in spontaneous movement. This decrease in activity progressed to moderate unresponsiveness to stimuli during the next two minutes. At that time the rat was extremely weak and could not walk but could crawl. The

Two rats died following IP injection (see Table 3). Rat 7 was injected twice, the second IP injection being 7 days after the first. Both rats exhibited a similar progression of clinical signs with no abnormalities noted for the first 5-6 minutes. The rats then became weak, could not walk, but could crawl. The severity of the clinical signs progressed during the next 6 minutes with the onset of and increase in labored breathing, tremors of the neck and back muscles followed by kicking of the backlegs. Death quickly followed the onset of violent, rapid (every 2-3 seconds) hindlimb kicking and apparent respiratory paralysis at 11-13 minutes postdosing.

c. Phrenic Nerve Stimulation in Animals Given Antx-A

In the study to assess phrenic nerve/diaphragm neuromuscular transmission, problems encountered initially included excessive hemorrhage in the first 3 rats and excessive stimulation and contraction of the thoracic limb with little evidence of diaphragmatic contraction noted.

Most of these problems appear to be corrected by altering the surgical approach. Less tissue trauma occurs and all the local major vasculature is avoided. This greatly decreases the amount of hemorrhage.

---

[1] Harvard Subminiature Electrode, Harvard Apparatus Company, Inc., South Natick, MA.

By using small cuff electrodes[1] and transecting the phrenic nerve anterior to the electrodes, it appears that the inadvertant stimulation of local neural and muscle tissue with resultant contraction can be avoided. The new approach and electrodes have only been used in three rats thus far. The surgical techniques involved will still require some practice before using this technique in formal studies of antx-a.

DISCUSSION

The work completed in the investigation of antx-a thus far would suggest that the toxin has a fairly steep dose-response curve. This is supported by the fairly narrow IV dose range that produced clinical signs ranging from fairly brief, moderate muscle weakness to death. The rapidity with which clinical signs appeared and death resulted following IV injection would suggest that antx-a is quickly distributed from the blood to its effector site(s). Intraperitoneally dosed rats required higher doses to produce clinical effects and experienced a longer time until onset of signs. The time to death with IP dosing was in agreement with other investigator's experimental findings. The differences in dose and time to clinical signs may potentially be explained in part by the polarity of antx-a preventing its rapid uptake from the peritoneal cavity by the vasculature.

No gross lesions were found during necropsy of the rats that died. No treatment-related microscopic lesions were found in tissues from the two rats that received the largest doses IV and IP (#2-IV; #7-IP).

FUTURE PLANS

In the next quarter, the technique to electrically stimulate the diaphragm via the phrenic nerves, will be perfected. The dosages employed in the preliminary study will serve as a guide for use in this site of action assessment. Approximately 30 ml of the "semipurified" antx-a used in this study remains and will be employed in the following aspects of the study to

maintain consistency. Once the technique is readily repeatable, antx-a will be administered IV at a known lethal dose (determined in the last quarter) and the effect on the diaphragm will be noted. Rapid onset of diaphragmatic and respiratory paralysis after dosing would suggest that the distribution of effector sites at least partially includes the peripheral nervous system. If diaphragmatic contraction continues unaffected, the suggestion would be that the antx-a effects on respiration are primarily in the central nervous system. We anticipate immediate respiratory paralysis which will not be particularly responsive to phrenic nerve stimulation. This would support a hypothesis that the peripheral myoneural junction is the principle site of action of antx-a.

Figure 1. Flow Diagram for and Clean up of "Semipurified" Anatoxin-A.

5.0 g Lyophilized cell

200 to 250 ml 95% Ethanol (pH 4.0)
stir 18 hr.

Centrifuge 3000 rpm 15 min

.45 um Filter paper

.20 um Filter paper

Bond Elut C_{18}

Dry down, reconstitute in 50 ml saline

Freeze

Table 1. Data for Rats Given "Semipurified" Anatoxin-A by Intravenous Administration.

Rat	Weight (in grams)	Volume of Toxin (in milliliters)	Dose (ml/kg)	Results
1	295.0	0.5	1.69	Survived
2	283.0	1.0	3.53	Died
3	302.7	0.8	2.64	Died
4	295.4	0.6	2.03	Survived
5	299.5	0.7	2.34	Died

Table 2
Usual Time Course of Effects in Rats Dosed Intravenously with Anatoxin-A

Time (Seconds Postinjection)	Effects
0–30	weakness, inability to walk, crawling
30–60	limp tail, arching of neck, kicking of backlegs
60–90	slight to moderate contraction of back muscles (sometimes involving the general musculature), increased frequency of kicking with backlegs, no evidence of respiration
*90	death

*Rat 5 died at 150 seconds postdosing. The onset of each of the above groups of clinical signs was delayed approximately 30 seconds, as compared to rats 2 and 3.

Table 3. Data for Rats Given an Intraperitoneal Injection of "Semipurified" Anatoxin-A.

Rat	Weight (in grams)	Volume of Toxin (in milliliters)	Dose (ml/kg)	Results
6	312.4	0.74	2.37	Survived
7	296.4	0.77	2.60	Survived
8	310.8	1.10	3.54	Died
9	355.4	1.10	3.10	Survived
10	349.4	1.15	2.29	Survived
*6	323.9	1.15	3.55	Survived
*7	324.0	1.20	3.70	Died

*Dosed twice, the second injection followed the first by 7 days.

BLUE-GREEN ALGAE (*MICROCYSTIS AERUGINOSA*) HEPATOTOXICOSIS IN A HERD OF DAIRY COWS

F. D. Galey, DVM[a], V. R. Beasley, DVM, PhD[a], W. W. Carmichael, PhD[b], G. Kleppe[c], DVM, S. B. Hooser, DVM[d], W. M. Haschek, BVSc, PhD[d]

Key words: Blue-green algae, Cyanobacteria, Microcystis aeruginosa, Hepatotoxicosis.

SUMMARY

Twenty cows from a dairy herd consisting of 60 adult, healthy, lactating Holstein dairy cows developed signs of anorexia, mental derangement, dehydration, recumbency and ruminal atony after the ingestion of water containing blue-green algae. Nine of the cows died. The algal bloom, which developed in a stagnant pond during hot, dry weather, was positively identified as the cyanobacterium Microcystis aeruginosa, a potentially hepatotoxic algae. One week after the incident, previously affected cows appeared healthy although liver-associated enzyme activities were elevated.

Intraruminal administration of the intact wet bloom to an apparently healthy 125 kg heifer was followed by hepatic necrosis and death. The liver was enlarged, friable and gunmetal blue in color with microscopically evident hepatocyte dissociation, degeneration and necrosis. The ingesta of the animal contained typical clumps of cells which were identified as M. aeruginosa.

The intraperitoneal (IP) administration of lyophilized cell material from that bloom to ICR Swiss male mice caused marked hepatic enlargement. The IP LD_{50} of the dried bloom was estimated to be 10 mg/kg.

A cyclic peptide toxin purified from the algae appears to be structurally similar to toxins from other previously characterized hepatotoxic blooms of M. aeruginosa.

Poisoning due to the blue-green alga (cyanobacterium) Microcystis aeruginosa has been reported in livestock from various parts of the world including Australia, South Africa, and Canada.[1,2,3] A cyclic peptide produced by toxic blooms of this alga causes massive hepatotoxicosis when ingested in sufficient amounts.[1,2,3] Potentially toxic blooms of Microcystis tend to form in water when dry weather and high nutrient concentrations are coupled with slow water movement.[1] Poisoning is more likely when animals have access to an area where wind or water flow have concentrated the bloom near the edge of a lake, pond or stream.

The present report describes: (1) environmental, clinical and serum chemistry findings associated with a naturally occurring outbreak of Microcystis aeruginosa toxicosis in a herd of dairy cattle; (2) the clinical, serum chemistry and pathologic findings in a heifer given the suspected algal bloom from the dairy farm; (3) the isolation and characterization of the toxin; and (4) a field diagnostic technique for confirming exposure to a Microcystis bloom.

Materials and Methods

Field investigation--On September 26, 1985, toxicologists from the National Animal Poison Control Center, College of Veterinary Medicine, University of Illinois at Urbana-Champaign investigated a suspected blue-green algae toxicosis which began one week previously in a herd of dairy cattle near Monroe, located in Green County of southern Wisconsin. The site was inspected and historical information was recorded. Serum samples for biochemical assessment (including serum albumin, alkaline phosphatase (AP), arginase, gamma glutamyl transpeptidase (GGT), aspartate amino transferase (AAT), bilirubin, blood urea nitrogen (BUN), creatine phosphokinase (CPK), creatinine, lactate dehydrogenase (LDH), calcium, glucose and electrolytes) were obtained from the tail veins of seven cows that had been clinically

affected, seven cows that had been exposed to the algae but were not clinically affected and three animals that had not been exposed to the algae. Twenty gallons of algal bloom (approximate dry weight of the cells was 20 g/l) were collected from the pond, overflow and drainage ditch for further study. Fresh and formalin fixed samples were sent (to W. W. Carmichael) for algal identification and toxin characterization.

Experimental protocol--Because postmortem examinations had not been performed on the cows that died in Wisconsin, a trial was conducted using an apparently healthy, 125 kg black Angus ruminant heifer in order to support the field diagnosis and document blue-green algae toxicosis in cattle. The equivalent of two grams (dry weight basis) of Microcystis cells per kilogram body weight was administered intraruminally to the heifer via a stomach tube. Eight liters of the wet bloom were divided into two doses given one hour apart in order to avoid excessive distension of the rumen. The dry weight dosage selected was similar to that which was experimentally lethal to sheep.[1] The animal was continuously observed for clinical signs. Samples for hematology and serum chemistry analysis were obtained from the jugular vein twice during the week prior to, immediately before and every 3 hours after dosing.

Gross necropsy was performed immediately after the heifer died. Tissues were fixed in 10% neutral buffered formalin, imbedded in paraffin, sectioned at 5 um and stained with hematoxylin and eosin. Samples of bloom and ruminal, omasal and abomasal contents were refrigerated and wet preparations were examined under a coverslip with a light microscope.

Algae and Toxin Identification--The cell material was freeze dried and sonicated to release the suspected water soluble toxin. The aqueous extract was then purified using gel permeation chromatography and reverse phase high performance liquid chromatography (HPLC).

An approximate intraperitoneal LD_{50} of the freeze-dried bloom for ICR Swiss male mice (15-25 grams) was determined. Body weights and liver weights as a percentage of body weights were obtained immediately after death.

High pressure liquid chromatography (HPLC) correlation profiles were studied for the purified Monroe toxin with two cyclic peptide toxins, AKERTOX[f] (a toxin produced by a Norwegian M. aeruginosa water bloom[4]) and the toxin produced by laboratory M. aeruginosa strain 7820. HPLC was done using an Alltech[g] C-18 column, 25 cm x 4.6 mm inside diameter with 10 micron packing and a liquid phase of 28% acetonitrile plus 10 mM ammonium acetate (pH 1.0) at a flow rate of 1 ml/min at 22 C. Toxins were detected with a Gilson Holochrome ultraviolet detector set at 240 nanometers. Ratios of amino acid constituents for the toxin were obtained by hydrolysis with 6N HCl precolumn derivatization with phenylisothiocyanate and analysis with a Waters Picotag HPLC system and UV detection at 254 nm.[f]

Statistical analysis--Clinical pathologic parameters of the groups were compared using the unprotected Least Significant Differences (LSD) Method[5] at a significance level (alpha = .05).

Results

Environmental findings--The weather had been hot and dry for several months prior to the incident and a thin, grainy, green film had formed on a stagnant, spring-fed pond (Fig 1). On September 17, 1985 wind had concentrated the bloom near the pond overflow pipe and it began to rain. By the next day, one inch of rain had fallen, filling the pond which then began to drain. A large amount of the thick, concentrated algal bloom was carried out of the pond and deposited along a shallow ditch in a pasture where lactating dairy cows were often kept.

Exposure history and clinical findings--On the evening of September 18, after the rain had subsided, 60 healthy adult lactating Holstein dairy cows (in all stages of lactation) were turned into the pasture. Twelve hours later, twenty cows were anorexic and three recumbent. Some of the affected cows had the green, grainy algal material on their backs, which, when sampled on September 19, was confirmed (by light microscopy) to consist of the same clumps of cellular material as found in the pond and outflow. The cows exhibited clinical signs resembling milk fever (anorexia, unresponsiveness, reluctance to move and occasional derangement). Recumbent cows rapidly became dehydrated. Ruminal atony and mild bloat developed.

The three cows that first went down had low pretreatment blood calcium concentrations of 6.4, 7.5 and 7.4 mg/dl, and initially responded to intravenous calcium treatment.[h] All of the animals were removed from the pasture. Ten other severely affected animals were later given calcium intravenously along with oral activated charcoal. Two of the animals that had earlier responded to the calcium treatment became recumbent and died later that day. Five more cows were dead the next morning and two additional animals died the following day. Unfortunately, postmortem examination was not conducted on any of the nine cows that died. One week after exposure the remaining affected animals appeared healthy and were returning to their normal feed consumption and lactation rates.

The experimentally dosed heifer became slightly less responsive seven hours after exposure to the toxic bloom and at eight hours postdosing, the calf was recumbent although it would reluctantly rise when stimulated. The heart rate increased from a predosing rate of 90 to 114 beats per minute (bpm); the respiratory rate increased from 42 to 66 breaths per minute and the rectal temperature rose from 38.5 C to 40.5 C. Profuse, watery diarrhea developed which continued until death. The heifer was anorexic, less

responsive, and weak. The rumen contraction rate slowed from one contraction per minute (predosing) to one contraction every three minutes.

The heifer's condition rapidly deteriorated during the subsequent two hours. The mucous membranes became pale and the capillary refill time was prolonged. The heart and respiratory rates continued to rise with the former exceeding 200 bpm. The calf became laterally recumbent, unresponsive to external stimuli, and died ten hours after dosing.

Serum chemistry findings--The dairy cows from the farm that had shown evidence of toxicosis had significantly elevated AP, GGT, AAT and LDH activities as compared to those from unaffected exposed and non-exposed cows (Table 1) seven days after exposure to the algae. The total bilirubin concentrations for three of the seven previously affected cows were marginally elevated. Serum electrolytes and renal parameters were all within normal ranges for cattle.

For the experimentally dosed heifer, the AP, arginase, GGT, AAT and LDH enzyme activities progressively increased after exposure (Table 2). In addition, the activity of creatine phosphokinase steadily rose to almost 3-4 times the predosing value. The total bilirubin concentration showed a slight increase late in the toxicosis. Glucose rose early from a predosing concentration of 88 mg/dl to 276 mg/dl at 8 hours and then dropped to 42 mg/dl ten hours after exposure. Other parameters were unchanged.

Gross pathologic findings--The liver from the heifer was markedly enlarged, purple-black in color and on cut section was friable and severely congested. The gall bladder contained dark red bile. The wall of the gall bladder was markedly thickened due to edema. This edema extended into adjacent pancreatic and mesenteric tissue. All mesenteric lymph nodes were enlarged (2 X normal size) and cortices bulged on cut section. Ingesta from the rumen, reticulum and abomasum was watery green with a grainy texture much

like the algal bloom itself (Fig 2). There was scant evidence of ingesta in the intestines. The jejunum contained a small amount of thick dark-red material. A small amount of clotted blood was present in the colon. The kidneys were slightly pale.

Histologic findings--ruminal, omasal, and abomasal contents from the heifer contained large numbers of clumped Microcystis aeruginosa cells similar to those seen in the pond (Fig 3).

In the liver there was severe hepatocellular dissociation, degeneration and necrosis involving the entire lobule except for a rim of periportal hepatocytes a few cells wide. The affected hepatocytes were widely separated by a large number of red blood cells with a few scattered neutrophils (Fig 4). Central veins were frequently obliterated or contained free hepatocytes. Severe subserosal edema was present in the gall bladder. The lymph nodes were edematous and red blood cells along with a mixed population of inflammatory cells were present in the sinuses. Hemorrhages were present in the lamina propria of the intestines.

Other lesions, presumably present before dosing and therefore not related to toxin administration, included moderate subacute multifocal interstitial nephritis, mild multifocal suppurative bronchiolitis and moderate enteritis.

Algae and toxin identification--The green clumps of cells were positively identified as Microcystis aeruginosa (Fig 5) based on microscopic morphology. When administered intraperitoneally to mice, the lyophilized bloom caused death with massive hepatic enlargement (liver weight was 9% of the body weight as compared to 5% for control) and dark red discoloration. The murine LD_{50} for the lyophilized bloom was determined to be 10 mg/kg.

The purified compound, like toxins previously purified and characterized from strain 7820 and AKERTOX, is a cyclic peptide and contains several

similar amino acids (equimolar ratios of alanine, arginine, glutamate, leucine and beta methyl aspartate).[j] The purified toxin from the Monroe bloom exhibited similar HPLC retention times as AKERTOX and the toxin produced by strain 7820 (Fig 6). Also, when equal amounts of Monroe toxin and one of the other toxins were combined, the resultant peak was doubled in size, symmetric and at a similar location. This purified material was shown to retain the toxicity of the lyophilized cells as it caused hepatic enlargement and death when injected intraperitoneally in mice.

Discussion

This report illustrates the potential hazard of M. aeruginosa as a hepatotoxin in dairy cattle. The diagnosis made following the field investigation was supported by experimentally reproducing of the syndrome.

The hot, dry weather and stagnant water apparently supported the growth of the toxic blue-green algae bloom as previously described.[1] The signs of anorexia, unresponsiveness, variable hyperthermia, recumbency, rumen atony, shock and death seen in the field and/or in the heifer dosed experimentally are consistent with those reported for sheep orally dosed with large amounts of Microcystis bloom.[1] These signs are also consistent with massive liver damage.

Elevations of AP, arginase, GGT and AAT activities all suggest a primary problem of liver damage. The elevations of CPK and LDH, which are less organ specific, could have been due to recumbency as well as to the liver damage.

The gross and light microscopic evidence of M. aeruginosa cells in the ingesta of the exposed animals is an important finding for two reasons. First, examination of wet preparations of ingesta by light microscopy at low to medium magnification can be easily and quickly used in the field to confirm bloom consumption. Second, the intact clumps of cells could be a source of prolonged exposure of the animal to the toxin throughout the

gastrointestinal tract. This evidence suggests, therefore, that attempting to remove the toxin, or prevent its absorption via activated charcoal adsorption, may be of potential benefit in the clinically exposed animal.

The intraperitoneal LD_{50} of 10 mg/kg estimated in mice for the lyophilized bloom material was lower than the approximate LD_{50} of 40 mg/kg found for mice using other M. aeruginosa blooms in this laboratory. The prominent hepatic enlargement was typical of experimental M. aeruginosa toxicosis in mice.[6]

Gross and histologic evidence of hepatic necrosis has been reported in sheep[1] and mice[3] exposed to M. aeruginosa blooms. The pathologic effects in the calf dosed in this study were consistent with those previous reports.

The HPLC correlation profile of the Monroe toxin indicates that it is very similar or identical to the hepatotoxins of strain 7820 (studied in this laboratory) and AKERTOX[i] isolated from another toxic M. aeruginosa bloom. The retention times and symmetrical peaks for the combined toxins provide further evidence that the toxins are similar. The initial amino acid profile of the Monroe toxin was also quite similar to that of previously characterized cyclic polypeptide hepatotoxins.[j] Yields from freeze-dried cells of the Monroe bloom consistently were about 4 mg of toxin per gram of cells. This compares with about 1 mg of toxin per gram of cells for the laboratory strain 7820 and is thought to explain the difference in toxicity between the two (i.e. 40 mg/kg for 7820 and 10 mg/kg for the Monroe toxic cells).

M. aeruginosa toxicosis occurs under conditions favorable to the growth and concentration of a toxic bloom, such as warm ambient temperatures, stagnant water, high water nutrient concentrations and wind. Toxicosis should be suspected when appropriate clinical signs, clinical pathologic and postmortem lesions of massive hepatic necrosis along with evidence of bloom consumption are present. This evidence may be apparent when animals have

access to a potentially toxic bloom of algae and/or when clumps of Microcystis cells found in the ingesta. For confirming a diagnosis, samples of the ingesta and bloom should be split and a small portion (i.e. 5 ml) preserved in an equal or larger volume of 10% neutral buffered formalin or dilute Lugol's solution and the remainder (i.e. 500 ml) refrigerated (not frozen) and rapidly shipped to a laboratory. These samples can then be used for positive alga identification and for proof of toxigenicity by mouse bioassay along with toxin isolation and identification.

At present, recommended therapy centers on prevention of absorption and support. Poisoned animals should immediately be removed from the source to prevent further exposure. Rumen lavage followed by oral administration of activated charcoal may reduce absorption of the toxin although the benefit of activated charcoal remains to be proven. Fluids, glucose and calcium given intravenously may benefit the clinically affected animal. Serum enzymes (especially those reflecting liver status) should be monitored.

[a]National Animal Poison Control Center
Department of Veterinary Biosciences
College of Veterinary Medicine
University of Illinois
Urbana, IL 61801

[b]Department of Biological Sciences
Wright State University
Dayton, OH 45435

[c]Monroe Veterinary Service, 1317-31st Avenue, Monroe, WI

[d]Department of Veterinary Pathobiology
College of Veterinary Medicine
University of Illinois
Urbana, IL 61801

[e]The authors thank Mr. Andrew M. Dahlem and Mr. Jeff Eschedor for their technical assistance. We acknowledge contracts DAMD 17-85-C-5241 and DAMD 17-83-C-4156 from USAMRDC which supported much of this work

[f]Krishnamurthy T, Carmichael WW and Sarver EW. Investigations of freshwater cyanobacteria (blue-green algae) toxic peptides. I. Isolation, purification and characterization of peptides from Microcystis aeruginosa and Anabaena flos-aquae. Toxicon, in press.

[g]Alltech, Deerborne, IL

[h]Cal Dextran II[R], Fort Dodge Laboratories, Fort Dodge, IA

[i]Krishnamurthy T, Carmichael WW and Sarver EW. Investigations of freshwater cyanobacteria (blue-green algae) toxic peptides. I. Isolation, purifications and characterization of peptides from Microcystis aeruginosa and Anabaena flos-aquae. Toxicon, in press.

[j]Krishnamurthy T, Hunt DF, Shubanowitz J, Carmichael WW, Missler S, Skulberg V and Codd G. Amino acid sequences of fresh water blue-green algal toxic peptides by fast atom bombardment tandem mass spectrometric technique. Proc. 34th Annual American Society Mass Spectrometry. Cincinnati, Ohio, June 9, 1986.

References

1. Jackson ARB, McInnes A, Falconer IR, et al. Clinical and Pathologic Changes in Sheep Experimentally Poisoned by the Blue-Green Alga (Microcystis aeruginosa). Vet Pathol 1984; 21:102-113.

2. Carmichael WW, Jones CLA, Mahmood NA, et al. Algal Toxins and Water-Based Diseases. CRC Critical Reviews in Environmental Control 1985; 15:275-313.

3. Carmichael WW. Algal Toxins. Advances in Botanical Res. V.12. ed. by C.A. Cullon. Academic Press, London. 1986; p. 47-101.

4. Berg K, Carmichael WW, Skielberg OM, Benestad C and Underdal B. Investigation of a toxic water-bloom of Microcystis aeruginosa (Cyanophyceae) in Luke Akersvatu, Norway. Hydrobiologia. (In press).

5. Steel RGD, Torrie JH. Principles and Procedures of Statistics, A Biometrical Approach, Second ed., 1980, McGraw-Hill Book Co.; 173-177.

6. Falconer IR, Jackson ARB, Langley J, et al. Liver Pathology in Mice in Poisoning by the Blue-Green Alga Microcystis aeruginosa. Aust J Biol Sci 1981; 34:179-187.

TABLE 1--Serum enzyme activities found to be significantly ($p<.05$) elevated in dairy cows seven days after ingestion of *M. aeruginosa* as compared to unaffected but exposed and nonexposed groups of cows (Mean ± S.D.).

Group	Alkaline Phosphatase	Gamma Glutamyl Transpeptidase	Aspartate Amino Transferase	Lactate Dehydrogenase
Exposed-showing clinical signs (n=7)	195.1±26.8	201.3±28.8	138.9±15.5	1996.6±77.9
Exposed-no clinical signs (n=7)	70.6±10.11	19.4± 1.7	56.1± 4.2	755.1±49.3
Not exposed (n=3)	123.0±49.5	16.0± 1.0	46± 4.7	787.7±59.5

TABLE 2—Serum enzyme activities in a heifer orally dosed with M. aeruginosa.

Sample	Alkaline Phosphatase	Gamma Glutamyl Transpeptidase	Aspartate Amino Transferase	Lactate Dehydrogenase	Creatine Phosphokinase	Arginase
Predosing						
4 days	213	26	63	1636	188	6
2 days	146	22	47	1264	92	1
At dosing	120	19	46	1067	181	2
Postdosing						
3 hours	122	16	49	1058	286	4
6 hours	151	35	65	1101	295	11
9 hours	253	59	ND	1744	359	180
10 hours	488	65	ND	2015	631	135

FIGURES SUBMITTED FOR PUBLICATION

Fig 1--The pond in Monroe, WI contained a green grainy film of blue-green algae that was wind concentrated to one side (A). The grainy material from the pond water in a 100 x 15 mm petri dish (B).

Fig 2--Photographs of concentrated blue-green algae (M. aeruginosa) bloom illustrating the pasty, granular appearance of the material from the pond (A) and from the bovine rumen 10 hours after exposure to the toxic bloom (B). 100 x 15 mm petri dish.

Fig 3--Characteristic cluster of M. aeruginosa cells from the pond (A). These clusters give the bloom its grainy appearance. There was a lucent calyx surrounding the clusters of round cells. Similar clusters from material obtained from the rumen of a heifer 10 hours after bloom ingestion (B). Wet preparation under coverslip; X 20.

Fig 4--Bovine liver from experimental M. aeruginosa toxicosis showing heptocellular dissociation, degeneration and necrosis. Affected heptocytes are separated by large numbers of red blood cells. Note disruption of lobular architecture which is most severe in the centrilobular region (left) with a lesser effect in the periportal area (right). Hematoxylin and eosin; x 20.

Fig 5--Scanning electron photomicrograph from a colony of Microcystis aeruginosa cells (each cell is about 5 u in diameter) from the algae bloom in Monroe; x 2500, SEM by A. S. Dabholkar.

Fig 6--The purified Monroe blue-green algae toxin has the same retention time and correlates well with previously isolated M. aeruginosa hepatotoxins AKERTOX (A) and strain 7820 (B). Arrow = injection time.

EFFECTS ON MALE MICE OF ONE OR TWO INTRAPERITONEAL DOSES OF A HEPATOTOXIN PRODUCED BY MICROCYSTIS AERUGINOSA

Randall A. Lovell, DVM, Val R. Beasley, DVM, PhD,
Wayne W. Carmichael, PhD, Andrew M. Dahlem, BS

INTRODUCTION

Microcystis aeruginosa, a freshwater cyanobacterium, produces a hepatotoxin which causes livestock and wildlife poisonings in both northern and southern temperate latitudes (1). Animal poisonings often follow ingestion of wind-aided accumulations of toxic cyanobacterial blooms along shorelines of ponds and lakes (2). Strains of Microcystis aeruginosa produce a cyclic heptapeptide, termed "microcystin," which is comprised of 2 variant L-amino acids, 3 invariant D-amino acids, a N-methyl α, ß-dehydroamino acid, and an extremely nonpolar 20 carbon ß-amino acid known as ADDA (3). The microcystin used in this experiment was derived from Microcystis aeruginosa laboratory strain 7820 and contained leucine and arginine as the 2 variant L-amino acids (3). Thus, "7820 microcystin" is chemically equivalent to cyanoginosin-LR (4). Although the exact mechanism of the hepatotoxic action of 7820 microcystin is unknown, it has been postulated that the hepatotoxin may destabilize the liver cytoskeleton (8,9). It has been suggested that animals given this toxin die from hemorrhagic shock due to acute liver hemorrhage (5).

The major purposes of this experiment were: (a) to establish a 24-hour IP LD_{50}, a "consistently lethal" IP dose, and a "consistently sublethal but toxic"

IP dose following a single administration of "purified" 7820 microcystin; (b) to assess whether a sublethal dose of 7820 microcystin provides a protective effect from a subsequent, "consistently lethal" (on a single-dose basis) IP dose of 7820 microcystin administered approximately 48 hours later; and (c) to compare liver and combined kidney weights, survival time, clinical signs, and gross necropsy findings of control mice with those of mice following 1 or 2 hepatotoxin administration(s).

MATERIALS AND METHODS

Doses were based on the weight of the 75% pure material, rather than on the weight of the toxin present. High doses of the contaminants present were tested in our lab by IP administration to mice, and did not demonstrate significant toxicity.

The day before dosing (Day 0), 0.15 ml of a stock solution (which correlated to 75.0 µg of 7820 microcystin of approximately 75% purity) was placed in a volumetric flask; physiologic saline was added to total 50 ml. The dosing solution was transferred into a 75 ml dark vial, capped, and stored in the refrigerator. Stability studies in our laboratory indicate that the hepatoxin does not significantly degrade for several days under these conditions.

The study used 60 male Balb/C mice weighing 20 to 35 g. After a 2-week acclimation period, each animal was randomly assigned to 1 of the following 6 beginning treatment regimens: tap water control or 20, 25, 30, 35, or 40 µg of 7820 microcystin/kg (n = 10 for each treatment group). Mice were weighed to the nearest 0.01 g immediately prior to dosing. The dosing solution concentration (1.5 µg 7820 microcystin/ml) was chosen so that correct IP doses could be delivered despite the small differences in dosage (5 µg 7820 microcystin/kg) between treatment groups.

On Day 0, all 60 mice were dosed. Mice that died within 24 hours (in treatment groups a, b, and c; Table 1) following a single microcystin administration were necropsied and comprised the single dose lethal response group. In addition, five randomly chosen control mice (treatment group f; Table 1) were killed and necropsied.

On Day 1, 12 mice were killed and necropsied to assess the sublethal effects of 7820 microcystin. These 12 mice were randomly chosen from the 6 beginning treatment groups containing greater than 5 survivors. These 12 mice (of treatment groups c, d, and e; Table 1) comprised the single dose sublethal response group. Five mice remained from each of the above-mentioned treatment groups for the repeated exposure study.

On Day 2, 48 hours after the initial dosing, all remaining mice were redosed using the same techniques and dosing solutions used on Day 0 to examine the effects of repeated 7820 microcystin exposures. The surviving hepatotoxin-treated mice were reweighed and given 40 µg 7820 microcystin/kg. The remaining 5 control mice were reweighed and again given tap water at a volume equivalent to that of the 40 µg 7820 microcystin/kg dose. All mice that died (if moribund mice were assessed as dead) within 24 hours following repeated microcystin administration were necropsied and comprised the second dose lethal response group (in treatment groups h, i, j, and k; Table 1). The 5 remaining control mice (treatment group g; Table 1) were killed and necropsied.

On Day 3, all mice still alive 32 hours after the second IP hepatotoxin dosing were killed and necropsied. These 3 mice (treatment group i; Table 1) comprised the second dose sublethal response group.

Monitoring for clinical signs was conducted for 10 hours after the Day 0 and Day 2 hepatotoxin dosings. At the start of Day 1 and Day 3, clinical signs were also recorded for the survivors.

All mice killed were subjected to cervical dislocation. Necropsies were performed within 30 minutes of death or euthanasia. Liver and combined kidney weights were obtained. The gallbladder was considered part of the liver weight; and perirenal fat was removed from the kidneys prior to weighing. Kidneys, liver, heart, lung, and spleen were preserved in 10% buffered formalin for histopathologic examination.

One mouse in the 35 µg/kg treatment group died unexpectedly during the night of Day 1. His organ weight results are not shown since more than 30 minutes likely elapsed after death before a necropsy was performed. This mouse was alive for the 24-hour LD_{50} calculations.

STATISTICAL ANALYSIS

To assess whether overall control observations could be combined, student's t-test ($\alpha = .05$) was used to compare liver and combined kidney weights expressed as percentages of live body weight (% BW) between the 2 control groups given 1 or 2 IP tap water doses (treatment groups f and g; Table 1). Covariate analysis will be used in the evaluation of the data generated to assess the interactions between dose, survival, organ weight, etc. Some of the methods described below represent preliminary data assessment only. Student's t-test ($\alpha = .05$) was used to compare survival times (following the most recent hepatotoxin administration) of the single dose lethal response group (combined treatment groups a, b, and c; Table 1) and the second dose lethal response group (combined treatment groups h, i, j, and k,; Table 1). A one-way analysis of variance ($\alpha = .05$) was used to compare liver and combined kidney weights (as % BW) between treatment groups within response groups (to compare treatment groups a, b, and c with... the single dose lethal response group; to compare treatment groups c, d, and e, within the single dose sublethal response group; and to compare treatment groups h, i, j, and k, within the second dose lethal

response group). A one-way analysis of variance (α = .05) and Duncan's New Multiple Range Test (α = .05) were used to compare liver and combined kidney weights (as % BW) from the 5 distinct response groups: (1) single dose lethal, (2) single dose sublethal, (3) control, (4) second dose lethal, and (5) second dose sublethal. The method of Miller and Tainter was used to calculate the $LD_{50} \pm$ its standard error (6).

RESULTS

Typical clinical signs observed in mice that ultimately died from the effects of 7820 microcystin included "normal behavior" for at least 20 minutes after the most recent hepatotoxin administration followed by reductions in activity and responsiveness, piloerection, pallor of ears and eyes, and sternal recumbency with intermittent attempts to rise. These were followed by a moribund appearance and death. Survival times are shown in Table 1. No seizures or hyperexcitability were observed. No clinical effects (other than equivocal mild depression and anorexia) were noticed in any control or sublethally dosed mice during the observation periods.

Necropsy Findings

No gross lesions were found in livers of the control and single dose sublethal response groups. Multifocal pinpoint reddened areas were seen in the livers of the 3 mice in the second dose sublethal response group. These were particularly evident near the margins of the liver lobes. Livers of the single dose lethal and second dose lethal response groups appeared similar on gross examination. The livers were greatly swollen, dark red in color and exuded blood when cut. No significant lesions were detected in the lungs, kidneys, heart, or spleen of any mice on gross examination.

Organ Weights

Table 1 illustrates the organ weight data obtained from the 11 treatment groups (a through k) and the 5 distinct response groups observed in this experiment. There were no statistically significant differences between the 2 control groups (treatment groups f and g; Table 1) for liver ($p = .068$) and combined kidney weights ($p = .5094$) (as % BW). Thus, the 10 control observations were pooled to provide the best estimate for these 2 parameters. From the initial dosing portion of this experiment, we established a 24-hour IP LD_{50} of 32.5 $\pm$ 1.2 µg for the 75% pure 7820 microcystin/kg, a 24-hour consistently lethal (10 of 10 died) single IP dose of 40 µg/kg and a 24-hour consistently sublethal (0 of 10 died) single IP dose of 25 µg/kg for male Balb/C mice. Interestingly, Table 1 indicates that each response group mean for liver weight (as % BW) significantly differed from those of the 4 other response groups when analyzed by the conservative Duncan's New Multiple Range Test ($\alpha = .05$). Control animals had the lowest liver weight (as % BW) and mice that died had the highest liver weights. Table 1 also indicates that combined kidney weights (as % BW) in both the lethal response groups were significantly greater than those in the control and sublethal response groups. Combined kidney weight data for the single dose lethal and second dose lethal response groups as a function of survival time are presented in Figure 1. Assessment of the combined kidney weight data (as % BW)) from the 10 mice with the shortest survival time (after the most recent hepatotoxin administration) revealed that 7 of them had values less than 1.56% (less than 2 standard deviations above the mean value for the control mice). All of the remaining 24 mice from either lethal response group had values for combined kidney weights (as % BW), greater than 1.56%.

A "protective effect" was seen in 3 of 5 mice which survived a 40 µg/kg IP dose of 7820 microcystin approximately 48 hours after being given 30 µg hepatotoxin/kg IP. This 40 µg/kg dose had been consistently lethal in 10 of 10 "virgin mice." Also, mean survival time (in minutes after the most recent hepatotoxin dosing) of the 14 mice comprising the second dose lethal response group was approximately 2.1 times greater than that of the 20 mice in the single dose lethal response group (392 vs. 189 minutes), although the difference was not statistically significant ($p = .1501$).

DISCUSSION

Although covariate analysis is needed, for preliminary use in this report, the data were statistically analyzed by response groups and not by treatment groups for the following reasons: (1) Each animal within a particular response group underwent the same pathophysiologic process (death or survival) following 1 or 2 doses of hepatotoxin. (2) There were no significant ($\alpha = .05$) treatment differences between treatment groups within a response group for liver weight (as % BW): single dose lethal response group, $p = .0735$; single dose sublethal response, $p = .9092$; second dose lethal response group, $p = .9309$ when analyzed by a one-way analysis of variance. (3) There were significant treatment differences between treatment groups within a response group for kidney weight (as % BW): single dose lethal response group, $p = .0047$; single dose sublethal response group, $p = .3983$; second dose lethal response group, $p = .3353$ when analyzed by a one-way analysis of variance. However, Figure 1 clearly illustrates that the differences in combined kidney weights (as % BW) were likely associated with differences in survival time between treatment groups. (4) There was no apparent association between decreasing hepatotoxin dosage and increasing survival time in the mice of the single dose lethal response group.

The mean survival time of the 3 mice that died from a single 30 µg hepatotoxin/kg dose was actually less than that of the 10 mice dying from a single 40 µg/kg 7820 microcystin dose (107 vs. 132 minutes). Thus, for the hepatotoxin dosages used in this experiment, it was felt that comparisons between response groups and not between treatment groups provided the most meaningful analysis of the data. This is likely to be demonstrated using covariate analysis.

An extremely steep LD_{50} curve was evident in this experiment. A similar dose-response curve was previously observed in sheep following intraruminal hepatotoxin administration (7). The single consistently sublethal IP dose in the mice of our experiment was 76.9% (25/32.5) of the LD_{50}. The consistently sublethal dose in the above-mentioned sheep study was greater than 90% of the LD_{50}. On a µg/kg basis, 7820 microcystin is one of the most potent compounds known to man (6).

Liver weight (as % BW) increased dramatically in this experiment. The single dose lethal response group had an average increase of 64% in liver weight (as % BW) when compared to the control group (8.45% of BW vs. 5.15% of BW). This marked increase in liver weight within hour(s) following hepatotoxin administration, appears to be caused primarily by hemorrhage and is likely to account for death in mice from hemorrhagic shock. The significant increase in liver weight (as % BW) for the single dose sublethal response group ($x = 5.59$), when compared to the control group ($x = 5.15\%$) (Table 1), suggests the following: (a) The IP single dose no effect level for "purified" 7820 microcystin is likely less than 20 µg/kg, even though no clinical signs or gross necropsy lesions were observed in the 5 mice of treatment Group e (Table 1); (b) the significantly higher liver weights of the second dose lethal response group as compared to the single dose lethal response group may be due to the additive effects of the single dose sublethal response upon the second dose lethal response.

Figure 1 suggests that the longer a mouse lives (especially between 70 and 200 minutes) following a lethal IP dose of hepatotoxin, the greater the opportunity for an increase in kidney weight (as % live BW). Since 7 of the 10 mice which died in less than 120 minutes (Figure 1) had "normal" kidney weights (less than 1.56% of the live body weight), then acute death caused by hepatotoxin administration is not necessarily associated with an increase in kidney weight in mice. The time course of changes in organ weights and gross necropsy findings suggests that the liver is the primary target organ and that the kidneys are secondarily affected following administration of 7820 microcystin. The mechanism by which the increase in kidney weight (as % liver BW) occurs is unknown. Histologic examination of kidneys, liver, and lung may provide a better understanding of the mechanisms by which these reported increases in organ weights occur. Despite the intuitively obvious trend in the data, the wide variation in times of death between animals treated with the hepatotoxin, caused simple linear and multiple regression analyses to fail to identify a statistically significant ($\alpha = 0.5$) trend in the data in Figure 1.

During a preliminary trial, a "protective effect" was seen in 2 mice which survived a 30 µg/kg IP hepatotoxin dose and 3 days later survived a 35 µg/kg or 40 µg/kg IP dose. In this experiment we again observed a "protective effect" in 3 of 5 mice which survived a 30 µg/kg IP hepatotoxin dose and 2 days later survived a 40 µg/kg dose. Similarly, survival of toxin LR-injected mice was improved when superimposed on carbon tetrachloride-induced sublethal hepatic damage.(10) However, in the present study, 3 treatment groups which survived the first IP hepatotoxin dosing (Day 0) did not have any mice that exhibited protection from the subsequent (40 µg/kg) 7820 microcystin dose (see treatment groups h, j, and k in Table 1). Large variations in survival time coupled with relatively small numbers of observations per response group may

have prevented the statistical recognition of an increase in survival time for the second dose lethal response group when compared to that of the single dose lethal response group. In single-dosed mice, 10 of 10 died after a 40 µg 7820 microcystin/kg dose. In contrast (counting preliminary and formal study) 4 of 18 mice "pretreated" with 7820 microcystin survived a subsequent 40 µg/kg dose. From the limited numbers in the repeated exposure portions of these experiments, it is suggested that a minimal "protective effect" from a "consistently lethal" dose of 7820 microcystin is likely to occur only in mice surviving a high (approximately 90% of LD_{50}) sublethal dose of hepatotoxin given 48 hours earlier. Deoxycholate, cholate, bromosulphophthalein and rifampicin given prior to hepatotoxin (microcystin) administration were found to prevent hepatocyte deformation in a dose-dependent manner (11), which suggested that a bile acid carrier was likely to be active in microcystin entry into hepatocytes. Damage to the bile acid active transport carrier system from the first hepatotoxin dose is therefore a possible mechanism for the "protective effect" observed in the present study.

In conclusion, following a single IP 7820 microcystin dose in mice, there is an extreme dichotomy of values for liver weights (as % BW). If mice survive a single hepatotoxin dose, liver weights ranged from 4.82 to 6.04% of live body weight. However, if mice died following a single hepatotoxin dose, liver weights ranged from 7.84 to 9.16% of live body weight. This dichotomy in liver weights, an extremely steep LD_{50} curve and no definitive trend for increasing survival time with decreasing dose of hepatotoxin suggests a tendency toward an all or none type response.

VRB:bcb/593
11b:11/17/86

1. Carmichael, W. W. Freshwater blue-grass algae (cyanobacteria) toxins--a review. In: The Water Environment: Algae Toxins and Health. Carmichael, W. W. (ed), Plenum Press, New York, 1981.

2. Carmichael, W. W., and Mahmood, N. A. Toxins from freshwater cyanobacteria. In: Seafood Toxins. Ragilis, E. P (ed), ACS Symposium Series 262, ACS Publ. Washington, D. C., 1984.

3. Carmichael, W. W. Personal Communication.

4. Botes, D. P., Wessels, P. L., Kruger, H., Runnegar, M. T. C., Santikarn, S., Smith, R. J., Barna, J. C. J., Williams, D. H. Structural Studies on Cyanoginosins -LR, -YR, -YA, and -YM, Peptide Toxins from Microcystis aeruginosa, J. Chem. Soc. Perkin Trans I, 2747-2748.

5. Falconer, I. R., Jackson, A. R. B., Langley, J., and Runnegar, M. T. Liver pathology in mice in poisoning by the blue-green alga Microcystis aeruginosa, Aust. J. Bio. Sci., 34(2), 179, 1981.

6. Osweiler, G. D., Carson, T. L., Buck, W. B., Van Gelder, G. A. Clinical and Diagnostic Veterinary Toxicology, Kendall Hunt Publishing Company, Dubuque, IO, 3rd (ed), 17-26, 198_.

7. Jackson, A. R. B., McInnes, A., Falconer, I. R., and Runnegar, M. T. C. Toxicity for sheep of the blue-green alga Microcystis aeruginosa, Toxicon, (Suppl) 3, 191, 1983.

8. Runnegar, M. T. C., and Falconer, I. R. The in vivo and in vitro biological effects of the peptide hepatotoxin from the blue-green alga Microscystis aeruginosa, South African Journal of Science (78) 363-366, 1982.

9. Runnegar, M. T. C., and Falconer, I. R. Effect of toxin from the cyanobacterium Microcystis aeruginosa on ultrastructural morphology and actin polymerization in isolated hepatocytes, Toxicon (24) (2), 109-115, 1986.

10. Adams, W. H., Stoner, R. D., Adams, D. G., Slatkin, D. N., Seigelman, H. W. Pathophysiologic effects of a toxic peptide from Microcystis aeruginosa, Toxicon (23) (2) 441-447, 1985.

11. Runnegar, M. T., Falconer, I. R. and Silver, J. Deformation of isolated rat hepatocytes by a peptide hepatotoxin from the blue-green alga Microcystis aeruginosa. Naunyn-Schmiedeberg's Arch. Pharmacol. (317):268-272, 1981.

TABLE 1

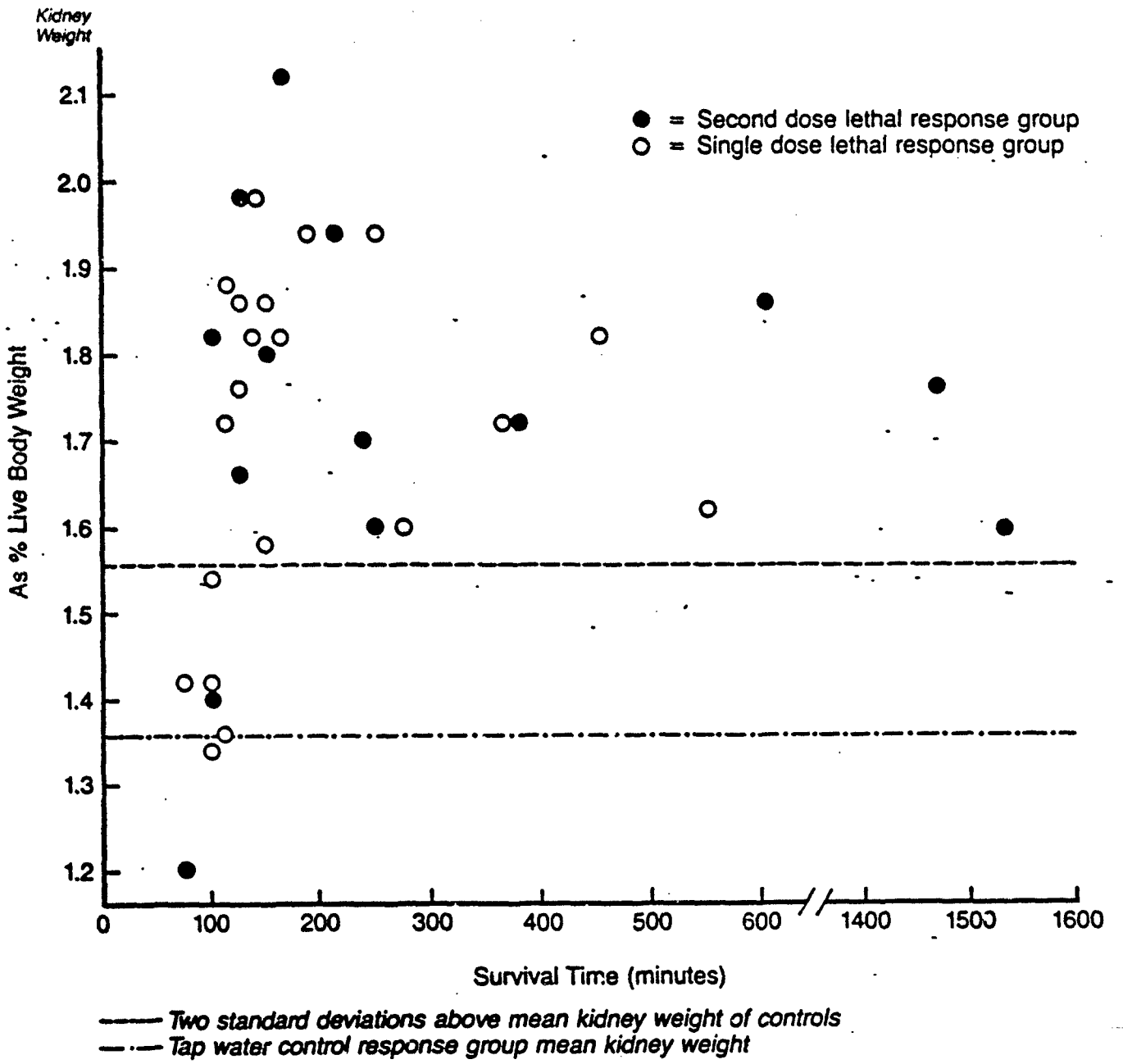
Treatment Group	IP Dose of 70% pure Hepatotoxin µg/kg Day 0	Day 2	No. Died After Most Recent IP Dose	No. Killed After Most Recent IP Dose	Mean Survival Time ± SD (Minutes)	Mean Liver Weight as % Live Body weight ± SD	Mean Kidney Weight as % Live Body Weight ± SD
Single Dose Lethal Response Group							
A	40	—	10	—	131 ± 32	8.66 ± .44	1.80 ± .15
B	35	—	7	—	308 ± 160	8.26 ± .21	1.67 ± .17
C	30	—	3	—	107 ± 8	8.24 ± .33	1.41 ± .12
Combined Group Response			20		189 ± 129	8.45 ± .40 D**	1.70 ± .20 B**
Single Dose Sublethal Response Group							
C	30	—	—	2	*1554 ± 105	5.40 ± .14	1.32 ± .10
D	25	—	—	5	*1572 ± 49	5.62 ± .25	1.36 ± .13
E	20	—	—	5	*1601 ± 50	5.59 ± .48	1.46 ± .15
Combined Group Response				12	1581 ± 56	5.59 ± .33 B	1.40 ± .15 A
Control Group							
F	0	—	—	5	*270 ± 80	5.31 ± .30	1.34 ± .14
G	0	0	—	5	*342 ± 9	4.99 ± .17	1.39 ± .04
Combined Group Response				10	306 ± 66	5.15 ± .28 A	1.36 ± .10 A
Second Dose Lethal Response Group							
H	35	40	2	—	227 ± 206	9.01 ± .96	1.46 ± .36
I	30	40	2	—	839 ± 965	8.93 ± .40	1.70 ± .13
J	25	40	5	—	529 ± 558	8.95 ± .71	1.83 ± .15
K	20	40	5	—	142 ± 58	8.74 ± .39	1.74 ± .26
Combined Group Response			14		392 ± 488	8.88 ± .54 E	1.72 ± .24 B
Second Dose Sublethal Response Group							
I	30	40	—	3	*1934 ± 6	6.67 ± .29 C	1.33 ± .06 A

* = Killed by cervical dislocation

** = Means with different letters were significantly different ($\alpha = 0.05$).

VRB:llb:bcb/593
11/17/86

Figure 1


Kidney Weight
As % Live Body Weight
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
● = Second dose lethal response group
○ = Single dose lethal response group
0
100
200
300
400
500
600
1400
1500
1600
Survival Time (minutes)
Two standard deviations above mean kidney weight of controls
Tap water control response group mean kidney weight

DETERMINATION OF ARGINASE ACTIVITY IN TWELVE TISSUES AND SERUM FROM MARKET WEIGHT SWINE BY A DIRECT COLORIMETRIC METHOD

Randall A. Lovell, DVM
Walter E. Hoffmann, DVM, PhD
Lisa A. Lund, DVM
Val R. Beasley, DVM, PhD

CONTENTS

INTRODUCTION

In all ureotelic organisms, arginase is an important hepatic enzyme that catalyzes the hydrolysis of the guanidino group of arginine to generate ornithine and liberate urea as an end product of nitrogen metabolism (1,2). The arginase catalyzed reaction is also the first in a series of reactions by which some microorganisms mobilize arginine as a nitrogen source(2). Manganese forms tight complexes with arginase, provides stability and functions in the catalytic center(2). Ornithine and lysine are potent competitive inhibitors of the reaction of arginine with arginase(1). In the rabbit liver, arginase has a molecular weight of 110,000 and is located in the cytosol(3). The half-life of arginase in the rat liver is around 96 hours(1,2).

Regulation of liver arginase can involve a change in either synthesis or degradation. Liver arginase activities increase in starved or glucocorticoid dosed animals due to reduced degradation of the enzyme. Provision of a diet rich in protein is associated with increased synthesis of arginase(1).

Although arginase has been shown to be almost exclusively liver specific in the ox, sheep, horse, rat and dog(3), no quantification of arginase activities of various body tissues and fluids could be found for swine.

MATERIALS AND METHODS

a. Animal Specimens

The tissues of six market weight (175-220 lbs.) Yorkshire and Yorkshire cross gilts were kindly donated to us. Four of the six gilts(2,3,4,6) had been spleenectomized and all six had been used earlier in various research trials. The gilts were fed a corn/soybean based diet. Before qualifying for use in the arginase study each gilt was required to: a) live more than 100 days and gain more than 125 lbs. after termination of their involvement in other trials b) be judged as clinically healthy immediately prior to the present study and c) bear no gross lesions observed during tissue collection. Immediately before euthanasia a blood sample was collected from the jugular vein, placed on ice, and allowed to clot. Serum was harvested following centrifugation at 5000 x g for 10 minutes at 4°C.

All gilts were killed by captive-bolt stunning followed immediately by exsanguination. Within 40 minutes of death of each gilt, the following tissues had been placed on ice in tightly closed plastic bags: liver, kidneys, adrenals, pancreas, lung, jejunal mucosa, heart (R&L ventricles), brain (cerebrum and cerebellum), salivary glands (parotid and/or maxillary), spleen (gilts 1 & 5 only), diaphragm (gilt 2 only) and quadriceps muscle. The tissues from gilt 6 were divided into two groups, "fresh" and "frozen".

b. Tissue Processing and Analysis

Following completion of tissue collection, all tissues from gilts 1 through 5 and the "frozen" tissues from gilt 6 were chopped into small

pieces, sealed in plastic bags and stored at -12°C. The "fresh" tissues from gilt 6 were then chopped into small pieces, rapidly frozen in liquid nitrogen and then blended into a fine homogenous powder using a tissue blender. This "fresh" tissue powder (gilt 6 only) was held on ice in a sealed plastic bag for same day analysis. Within two days of tissue collection, the frozen tissues from gilts 1 through 6 were placed in liquid nitrogen and blended into a fine homogenous powder. The powdered tissues were sealed in plastic bags and returned to the -12°C freezer for storage. For all gilts, serum, quadriceps, diaphragm, spleen and salivary gland samples were analyzed for arginase activity 12 days after tissue collection; adrenal, brain, lung and heart were analyzed on day 13 and kidney, liver, pancreas and jejunal mucosa were analyzed on day 14.

On the day of arginase analysis one gram of the frozen tissue powder was weighed into a tissue pulverizer. Nine ml of physiologic saline was added to the tissue to aid in its disruption by manual force using a glass rod. After complete disruption of visible tissue structure, the tissue homogenate was poured into a test tube. The tissue homogenate was centrifuged at 4°C for 10 minutes at 500 x g, for removal of erythrocytes, nuclei, connective tissue and residual intact cells(4). For all tissues, except liver, kidney and pancreas, 20 ul of this tissue homogenate supernatant was used in the arginase analysis. For kidney and pancreas a 10:1 and for liver a 50:1 dilution of the supernatant with saline was required to obtain activity in the range of our standards. Reagents were prepared and the assay performed according to the method of Mia & Koger(5).

c. <u>Computation of Activity</u>

Simple linear regression analysis of the five standard curves run during days 12, 13 & 14 of this study provided five estimates of the

slope and y intercept.

To convert mean absorbence into IU/g for each specimen, the following determinations and formulae were used:

Mean absorbence - the average of each sample run in triplicate and averaged over duplicate blanks.

A mean y intercept of .0198 was calculated from the five standard curves

$\frac{1 \text{ m mole / L}}{.190 \text{ absorbence units}}$ = slope of the calibration curve which was calculated from the five standard curves

$\frac{50 \text{ IU / L}}{1 \text{ m mole / L}}$ = conversion factor of m mole / L to enzyme units under these test conditions

$\frac{L}{x \text{ grams of tissue}}$ is a conversion factor to allow for the amount of tissue analyzed and is calculated from adding 9 mls of physiologic saline to one or less grams of tissue so that that x = 111.1 for brain, heart, lung, spleen, diaphragm, quadriceps, salivary glands, adrenal and jejunal mucosa; x = 11.11 grams tissue per liter for kidney and pancreas, due to the necessary 10:1 dilution and x = 2.22 grams tissue per liter for liver, due to the necessary 50:1 dilution.

According to the definition of international units (IU) for enzymes, 1 IU of arginase would produce 1 u mole of ornithine/minute. Thus an ornithine standard of 1 m mole / L is equivalent to 1 m mole / L X 1,000 u mole/ m mole/ 20 minutes = 50 u mole per minute per liter = 50 IU / L of serum under the test conditions(5).

Therefore, [mean absorbence - y intercept] X

$$\frac{1 \text{ m mole / L}}{.190 \text{ absorbence units}} \times \frac{50 \text{ IU / L}}{1 \text{ m mole / L}} \times \frac{L}{x \text{ grams of tissue}} = \text{IU / g}$$

To convert mean absorbence into IU / L for each serum sample, the following formulae were used:

$$[\text{mean absorbence - y intercept}] \times \frac{1 \text{ m mole / L}}{.190 \text{ absorbence units}} \times \frac{50 \text{ IU / L}}{1 \text{ m mole / L}} = \text{IU/L}$$

RESULTS

The arginase calibration curve prepared with ornithine standards was calculated to have a slope of .190 ± .0146 absorbence units per m mole per liter and a y intercept of .020 ± .031 absorbence units. All five standard curves had a correlation coefficient of greater than .996.

The mean, standard deviation (SD) and range of the arginase activity in twelve tissues (IU/g) and serum (IU/L) from the six gilts are presented in Table 1.

Table 1

Tissue	Mean ± SD (IU/g)	Range (IU/g)	n
Liver	30.64 ± 22.95	12.33 - 67.47	6
Pancreas	4.89 ± 2.00	2.34 - 8.33	6
Kidney	2.91 ± 1.42	1.01 - 5.22	6
Salivary Gland	.44 ± .28	0.07 - 0.88	6
Jejunal Mucosa	.42 ± .14	0.21 - 0.57	6
Quadriceps	.077 ± .080	-0.05 - 0.17	5
Lung	.037 ± .107	-0.04 - 0.24	6
Brain	.004 ± .015	-0.01 - 0.03	6
Adrenal	.003 ± .068	-0.07 - 0.10	6
Spleen	.002 ± .067	-0.05 - 0.05	2
Heart	- .007 ± .023	-0.03 - 0.03	6
Diaphragm	.026		1
Serum	- .10 ± 6.35*	-9.76 - 5.24*	6

* = IU/L

DISCUSSION

No data is presented from the Day 1 analysis of the "fresh" tissues of gilt 6 for the following reasons: (a) this was the first time the assay was run in our laboratory for pigs; (b) the correlation coefficient of the standard curve was less than our acceptance limit (.985); (c) the liver, pancreas and kidney absorbence readings were greater than the highest absorbence reading on the standard curve; (d) the difference between absorbence of the reagent blanks was 2 1/2 times greater than our acceptance

limit (.016). This "practice run" nevertheless proved invaluable, since we learned the intricacies of the method and which tissues would need to be diluted and by roughly what amount for the analyses on Days 12, 13 and 14.

The twelve tissues collected from the 6 pigs in this study contained arginase activity in the following descending order: liver >> pancreas > kidney >> salivary gland = jejunum >> quadriceps, lung, diaphragm, brain, adrenal, spleen and heart.

Gilt 6 was the only animal in this study with more arginase activity per gram of kidney than per gram of pancreas and the only gilt with significant (> .06 IU / g) arginase activity in lung tissue. These results were present in both the fresh (Day 1) and frozen (Days 13 and 14) tissue analysis of Gilt #6. From Table 1 it is evident that only the liver, kidney and pancreas contain appreciable amounts of arginase. The liver contains approximately 6 times more arginase activity per gram of wet weight tissue than the pancreas and approximately 10 times more than the kidney. The salivary glands and jejunal mucosa contain minimal amounts of arginase activity. The large variation seen in salivary glands could be due to differences between maxillary and parotid glands as we collected parts of both during tissue collection. The quadriceps probably contains a very minute amount of arginase activity since 4 of the 5 samples had a mean activity of between .062 and .17 IU/g while the fifth sample showed a negative mean activity. The serum, brain (cerebrum and cerebellum), adrenal and heart (R&L ventricles) contained inconsequential amounts of arginase activity in the swine of this study. Why the lung tissue of gilt 6 was the only one to show significant arginase activity is unknown. The activity observed was, however, only a very small percentage of that found in the liver, kidney and pancreas. From the very limited sample size it appears that the spleen and diaphragm also contain negligible amounts of arginase activity.

Arginase activity appears to be much lower in swine liver (30.64 IU/g) in comparison to the five species (dog, rat, horse, ox and sheep) in which its activity has been reported. In Boyd's review (3), liver arginase activity ranged from 110.0 IU/g in sheep to 445.0 IU/g in the dog. Despite this lower activity, the results of this study indicate that arginase activity in swine could easily be called "liver specific." Although renal and/or pancreatic damage could contribute to or cause a moderate elevation in serum arginase activity in swine, liver damage is by far the most likely source of elevated arginase activity in swine, especially if one takes organ weights into account.

REFERENCES

1. Harper, H. A., Rodwell, V. W., Mayes, P. A. Review of Physiological Chemistry. Lange Medical Publications. 1979. Edition 17. pp. 87-95; 402-404, 409.

2. Zubay, G. Biochemistry. Addison-Wesley Publishing Company, Inc. 1983. pp. 239, 888, 965.

3. Boyd, J. W. The Mechanisms Relating to Increases in Plasma Enzymes and Isoenzymes in Diseases of Animals. Veterinary Clinical Pathology (12) (2). pp. 9-24. 1981.

4. Cacciatore, L. and Antoniello S. Arginase Activity of Mouse Serum and Liver Tissue in Some Conditions of Experimental Liver Damage. Enzymologia (41) (2). pp. 112-120. 1971.

5. Mia, A. S. and Koger, H. D. Direct Colorimetric Determination of Serum Arginase in Various Domestic Animals. Am. J. Vet. Res. (39) (5). pp. 1381-1383. 1978.

OVERVIEW OF FIRST YEAR STUDIES IN GILTS FOLLOWING HEPATOTOXIN ADMINISTRATION

Randall A. Lovell, DVM
Val R. Beasley, DVM, PhD
Gregg Lundeen, PhD
Ken Holmes, PhD

CONTENTS

INTRODUCTION

Suspected poisoning by freshwater algae was reported over a century ago.[1] Livestock and wildlife deaths due to consumption of toxic blue-green algal bloom are most frequently reported in northern and southern temperate zones.[2] Of the algae reported to produce toxic effects, Microcystis aeruginosa is probably the most frequent offender.[3]

The major goals of the first year of this study were a) to gather preliminary physiologic, pathologic, clinical pathologic and toxicologic information in swine following intravenous administration of purified 7820 microcystin, b) to become proficient in placing catheters in the left atrium, pulmonary artery, aorta and inferior vena cava in anesthetized gilts, c) to purchase and learn the intricacies of operating a state-of-the-art blood gas and electrolyte machine and a cardiovascular analyzer unit, and d) to develop techniques whereby the temperature pulse decay probe system can be safely utilized in unanesthetized animals.

MATERIALS AND METHODS

Two littermate gilts were dosed with hepatotoxin to gather initial dose-response and pathophysiologic information. One gilt was dosed orally with whole algal cells and another was dosed intravenously with "purified" 7820 microcystin.

Four gilts have been utilized in the temperature pulse decay (TPD) probe research. While under anesthesia, two gilts were implanted with TPD probes in the liver and kidney; and blood flow was monitored before and after hepatotoxin administration. Two gilts were involved in developing techniques aimed toward using the TPD probes in awake animals.

Experiments were conducted on 11 healthy gilts (Table 1) weighing 24.0 to 55.5 kg in order to evaluate hemodynamic and blood chemistry alterations induced by 7820 microcystin. Blood gas, serum chemistry and complete blood count (CBC) parameters were measured and clinical signs observed in each gilt. Hemodynamic measurements included aortic systolic, diastolic and mean pressures; left atrial, pulmonary artery and jugular vein mean pressures and cardiac output. Monitoring of heart rate and ECG was also conducted.

Sixteen and eighteen gauge Tygon^{R} catheters for hemodynamic monitoring were surgically implanted in these 11 gilts while under halothane anesthesia using methods previously described.[4,5] Before qualifying for hepatotoxin dosing, these 11 gilts were required to be at least two weeks post-thoracic surgery, 6 kg heavier, and healthy on physical examination. On the day of dosing, all gilts had their subcutaneously buried catheters exteriorized following local infiltration of 2% lidocaine. At least 2 1/2 hours elapsed between the lidocaine injections and iv hepatotoxin administration. Fifteen to eighteen ml of a 7820 microcystin-ethanol-physiologic saline solution was injected into the pulmonary artery over a 40-90 second period.

RESULTS

In the TPD probe study using anesthetized gilts, liver perfusion rapidly declined approximately 10 minutes after toxin administration and approximately 10 minutes prior to an abrupt fall in arterial blood pressure. In contrast, local kidney perfusion appeared to be directly correlated with changes in arterial blood pressure.

Striking hypoglycemia, life-threatening hyperkalemia and markedly elevated phosphorus concentrations were revealed by analysis of serum samples collected at the time of death in the seven catheterized gilts which died following hepatotoxin administration. Elevated serum bile acid concentrations appear to be the first indicator of liver damage. Elevations in creatinine phosphokinase, aspartate amino transferase (SGOT), arginase and total bilirubin concentrations consistently followed the rise in bile acids. Table 2, from gilt # 1583, typifies above mentioned increases/decreases in parameter values. A slight decrease in platelet numbers was observed in gilts 1261 and 1560.

Although the time course and degree of changes in blood-gas parameters varied, the direction of change was consistent in the seven catheterized gilts that died following 7820 microcystin administration. Arterial blood pH, $PaCO_2$, base excess, $-HCO_3$, and total CO_2 decreased in a manner highly suggestive of metabolic acidosis. Body temperature and PaO_2 levels appear to increase with time after dosing.

Every catheterized gilt which died following hepatotoxin administration developed marked hyperpnea, lethargy, cool extremities, cyanotic mucous membranes and apparent abdominal pain with multiple vomiting/retching episodes often preceded by bruxism (teeth grinding).

DISCUSSION

The results of changes in hemodynamic parameters will hopefully be presented in our next quarterly report. An "artificial spike" has led to a complete reevaluation of previously reported aortic systolic and pulse pressures. We believe further review of this preliminary hemodynamic data is needed before presenting an interpretation.

Despite minor difficulties (result interpretations, recovery from surgery and learning to use new equipment) much has been accomplished in the first year of this project. From these preliminary gilts we have a very good idea of the consistently lethal and consistently toxic sublethal iv dose of purified 7820 microcystin which will be used in our formal study. We believe that the liver is the primaryy target organ of 7820 microcystin and that pigs die from a combined effect of hemorrhagic shock following pronounced centrilobular hepatic hemorrhage, hypoglycemia, and hyperkalemia. We now have an adequate supply of > 95% pure 2780 microcystin, have worked out several problems in surgical techniques and have purchased state-of-the-art computerized equipment. Finally, we believe that SPF gilts will provide a highly satisfactory model for studying the effects of a purified toxin produced by the blue-green alga, Microcystis aeruginosa.

Table 1

Pig #	Dosage of Hepatotoxin (ug/kg)	% Purity Hepatotoxin	Survival Time (minutes)	Weight (kg)
863	492	75	47	29.8
807	64	75	*1240	30.7
874	128	75	257	43.2
1061	96	75	195	30.0
1032	64	75	1046	31.1
1193	0	NA	*1600	28.6
1261	64	75	510	55.5
1560	32	>95	243	28.75
1583	24	>95	325	24.0
1662	16	>95	*1600	24.75
1672	16	>95	*1600	24.5

*Killed by electrocution

Table 2

Time	Bile Acids (u/l)	CPK (u/l)	Arginase (IU/l)	Total Bilirubin (mg/dl)	SGOT (u/l)
Predose	4.8	627	0	0.2	47
30 min.	9.3	615	1	0.2	45
60 min.	6.2	537	1	0.3	47
120 min.	55.8	649	8	0.5	68
240 min.	60.7	1164	460	0.6	451
325 min. (term)	110.0	3100	1432	1.1	2090

Time	Glucose (mg/dl)	Potassium (meq/l)	Phosphorus (mg/dl)
Predose	89	4.0	8.3
30 min.	108	3.3	8.0
60 min.	135	3.4	8.0
120 min.	83	4.2	8.5
240 min.	49	4.8	9.4
325 min. (term)	5	9.1	13.5

REFERENCES

1. Francis, G. Poisonous Australian Lake. Nature (London) 18, 11-12, 1878.

2. Carmichael, W.W. Freshwater blue-green algae (cyanobacteria) toxins--a review. In: The Water Environment: Algae Toxins and Health. Carmichael, W.W. (ed.), Plenum Press, NY, 1981.

3. Gishi, S. and Watanabe, M.F., Acute Toxicity of Microcystis aeruginosa and its Cardiovascular Effects. Env. Res. 40, 518-524, 1986.

4. Lorenzana, R.M., Beasley, V.R., Buck, W.B., Ghent, A.W., Lundeen, G.R., Poppenga, R.H. Experimental T-2 toxicosis in swine. I. Changes in cardiac output, aortic mean pressure, catecholamines, 6-keto-PGF_1 alpha, thromboxane B_2, and acid base parameters. Fund. Appl. Toxicol. 5:879-892, 1985.

5. Lundeen, G.R., Manohar, M., Parks, C.M. Systemic distribution of blood flow in swine while awake and during 1.0 and 1.5 MAC isoflurane anesthesia without or with 50 percent nitrous oxide. Anesthesia and Analgesia. 62(5):499-512, 1983.

THE EFFECTS OF 7820 MICROCYSTIN, A CYCLIC HEPTAPEPTIDE TOXIN FROM MICROCYSTIS AERUGINOSA, IN THE RAT

Stephen B. Hooser, Wanda Haschek-Hock, Val R. Beasley

CONTENTS

INTRODUCTION

The hepatotoxicity of the cyclic heptapeptide produced by the blue-green alga, Microcystis aeruginosa has been documented from field cases involving sheep and cattle, has been implicated in human toxicoses following growth in reservoirs, and has been experimentally reproduced by dosing sheep, mice, and rats with whole cells or crude cell extracts.[1-8] However, the pathogenesis and exact mechanism of action are not known. It has been hypothesized that liver damage, which begins centrilobularly and progresses periportally, may be due to venous hypertension caused by massive pulmonary embolism[7] or due to a direct hepatotoxic effect. Death is postulated to be caused by hypovolemic shock associated with hepatic necrosis and hemorrhage or due to pulmonary embolism and subsequent venous hypertension, reduced cardiac output, and circulatory collapse.

For our studies, rats and mice were chosen as test animals because of their uniformity and their convenient size and cost. Also, their normal values over a wide range of parameters and their responses to a wide variety of substances have been established, allowing comparison to the effects of the toxin being studied. In addition, many in vitro procedures utilizing rat hepatocytes in cell culture and suspension have been developed which will be utilized in the course of future experiments.

In the present study, we characterized the acute toxicity of the purified heptapeptide hepatotoxin, microcystin, produced by the laboratory strain 7820 of Microcystis aeruginosa with respect to clinical signs and morphologic changes. We compared the toxicity of purified hepatotoxin; (a) in male and female rats; (b) at lethal and supralethal doses; and (c) in mice and rats. In addition, we characterized the sequential morphologic and clinical pathologic changes occurring in male rats with the hepatotoxin at the lowest dose found to produce 100% lethality (160 ug/kg).

MATERIALS AND METHODS

1. Animals

Male and female, virus-free, 175 to 200 g Sprague Dawley rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and allowed to acclimate for two weeks or longer before use. Adult, female, Balb/C, mice, raised in our own colony, were also used. Animals had free access to a commercial lab chow and water, and were maintained on a 12-hour light-dark cycle.

Rats and mice were randomly assigned to the various dose groups immediately before dosing and given free access to water until termination of the study.

2. Pathologic examination

Animals dying spontaneously or killed following treatment were immediately necropsied. Animals were anesthetized with ether and killed by exsanguination. The liver, kidneys (with adrenals), and spleen were weighed. Lungs were fixed by intratracheal instillation of 10% neutral buffered formalin. Sections of liver, lung, kidney, spleen, and thymus from all animals as well as brain, heart, adrenal gland, small and large intestine, pancreas, urinary bladder, skeletal muscle, and eyes of rats from the acute toxicity study were fixed by immersion in 10% neutral buffered formalin. The tissues were then processed routinely, embedded in paraffin, cut at 4 to 6 um, stained with hematoxylin and eosin and examined by light microscopy.

3. Acute toxicity of and LD_{50} determination for 7820 microcystin in rats

All animals were weighed immediately prior to use. 7820 Microcystin from strain 7820 of Microcystis aeruginosa, of approximately 95% purity, was provided by Dr. Wayne Carmichael of Wright State University. Male rats were given an intraperitoneal injection of 7820 microcystin, dissolved in 0.9% NaCl at the following doses (number of rats in parentheses); 20 (3), 40 (3), 80 (3), 120 (3), 160 (8), 180 (3), 200 (3), 240 (2), and 400 ug/kg (3). The actual volumes of toxin and saline administered ranged from 0.1 to 1.0 ml. Animals were then observed for clinical signs of toxicosis for at least 24 hours. Similarly, female rats (three per group) were given an ip injection of 7820 microcystin at 0, 40, 80, 120, or 160 ug/kg. Animals dying spontaneously were immediately necropsied. The remaining animals were killed by ether anesthesia and necropsied five days after treatment. Male rats dosed with 20 ug/kg of the toxin were killed and necropsied 12 days post dosing to serve as rough controls.

In addition, the clinical course and toxic effects of very high doses of 7820 microcystin were determined in male rats. Rats (three per group) were given a single ip injection of 7820 at the following doses: 0 (2 ml of the vehicle, 0.9% NaCl) 400, 800, or 1200 ug/kg. All animals were necropsied immediately after death, the controls being killed 24 hours after saline administration.

4. Sequential morphologic and serum chemistry alterations in male rats

Rats were given an ip injection of 160 ug/kg of 7820 microcystin dissolved in 0.9% NaCl and killed and at the following times (number of animals in parentheses): 5 (2), 10 (2), 20 (5), 30 (5), 40 (5), 50 (2), and 60 (7) mins, and 3 (5), 6 (5), 9 (5), 12 (5), 18 (2), or 24 (2) hours. Control rats were injected ip with 0.5 ml of 0.9% NaCl and killed at 60 mins (3), and 12 (3) and 24 (3) hours. Blood samples were collected by cardiac puncture and immediately placed in serum tubes or tubes containing EDTA as an anticoagulant, and put on ice. Clotted serum samples were centrifuged within one hour, the clot was removed, and the samples were frozen until analyzed. Serum chemistry evaluation included: creatinine (mg/dl), blood urea nitrogen (BUN, mg/dl), total protein (T.P., gm/dl), albumin (gm/dl), calcium (mg/dl), phosphorus (mg/dl), sodium (mEq/dl), potassium (mEq/dl), chloride (mEq/dl), glucose (mg/dl), alkaline phosphatase (ALP, u/L), alanine aminotransferases [(ALT)=serum glutamic-pyruvic transaminase (SGPT)] (u/L), gamma glutamyl transferase (GGT, u/L), total bilirubin (mg/dl), albumin to globulin ration (A/G) and sodium to potassium ratio.

5. Acute toxicology and sequential morphologic alterations in mice dosed with 7920 microcystin

Female mice were given an ip injection of 100 ug/kg of 7820 microcystin dissolved in 0.9% NaCl, and killed (2 per group) at 15, 30,

60, and 90 minutes, with three mice surviving the 90 minute period necropsied immediately following spontaneous death.

6. Statistical analysis

Serum chemistry data were analyzed using a one-way analysis of variance[b].

RESULTS

1. Acute toxicity of and LD_{50} determination for 7820 microcystin in rats

Male rats dosed at 20, 40, or 30 ug/kg and female rats dosed at 40 ug/kg did not show any abnormal clinical signs and had no gross or histologic lesions. All animals which showed clinical signs died and had similar gross and histologic lesions irrespective of sex or dose. Clinical signs in affected animals were limited to severe depression and lethargy which began several hours after toxin administration and progressed in severity until the time of death. The time from injection of the hepatotoxin to death varied from 20 to 32 hours in males and females and was independent of dose. Deaths (number which died/number treated) in males were as follows: 120 ug/kg, 2/3; 160 ug/kg, 7/8; 180 ug/kg, 3/3; 200 ug/kg, 3/3; 240 ug/kg, 1/2; and 400 ug/kg, 3/3. Deaths in females were as follows: 80 ug/kg 1/3; 120 ug/kg, 2/3; 160 ug/kg, 3/3.

Rats given at very high doses of 7820 microcystin [400, 800, or 1200 ug/kg (3 animals per group)] died 6 to 8 hours following administration. Clinical signs of toxicosis consisted of extreme depression and lethargy beginning 4 to 5 hours after dosing and progressing until death. The livers from all rats which died after administration of the hepatotoxin were markedly dark red and appeared enlarged, particularly in those receiving higher doses of the toxin (Table 1).

---

[b] SAS Institute, SAS Circle, Cary, NC.

Microscopic examination of the liver revealed severe centrilobular and midzonal necrosis which was characterized by severe disassociation, degeneration, necrosis, and loss of hepatocytes within each lobule except for a rim of periportal hepatocytes 3 to 6 cells wide. Some of these periportal hepatocytes appeared normal, while others showed signs of degeneration (pyknotic nuclei and eosinophilic cytoplasm). Degenerating and necrotic hepatocytes in centrilobular and mid-zonal regions were separated by numerous erythrocytes and abundant granular to homogeneous material (cellular debris). Moderate numbers of neutrophils infiltrated the affected areas. Occasional degenerate and necrotic hepatocytes were seen in central veins.

Microscopic examination of the kidney revealed that glomerular capillaries were frequently dilated and contained small plugs of eosinophilic, finely fibrillar to homogeneous material which was often finely stippled with basophilic deposits. Cortical, tubular epithelial cells were moderately vacuolated in many areas. Numerous tubules within the cortex were mildly to moderately dilated, contained moderate amounts of homogeneous, basophilic material. With very high doses there was moderate tubular necrosis characterized by numerous, multifocal areas of epithelial degeneration and necrosis with small amounts of luminal necrotic debris. Many tubules within the cortex contained moderate amounts of homogeneous basophilic material.

On microscopic examination of the lung, many capillaries and somewhat larger vessels were widely dilated and contained eosinophilic globular debris or intact cells which were often enmeshed within an eosinophilic, finely fibrillar material. These cells, which appeared to be hepatocytes, were large and rounded with abundant eosinophilic cytoplasm and small, dense, dark, centrally located (pyknotic) nuclei.

2. Sequential morphologic and serum chemistry alterations in male rats administered 7820 microcystin

Beginning approximately 40 to 60 minutes after treatment, the livers from all hepatotoxin treated rats were very dark red and appeared enlarged. Although liver weights varied throughout the study, after 40 to 60 min., the livers of all microcystin treated rats grossly appeared larger. Over the 24 hr. time period, the liver weights (as % of body weights) from treated rats were significantly greater than those of the saline treated controls. (Figure 1). No other gross lesions were noted at any time, however, kidney weights (as % of body weights) began to increase at approximately 3 hours postdosing. This increase continued to the end of the study (Figure 2).

No microscopic changes were seen at 5 or 10 minutes following treatment. In the liver at 20 mins, in the liver there was mild disassociation and rounding up of centrilobular hepatocytes with mild sinusoidal congestion. A few scattered hepatocytes were shrunken with dark eosinophilic cytoplasm and clumping of nuclear chromatin. By 30 mins there was moderate centrilobular hepatocyte disassociation with mild hemorrhage involving the majority of centrilobular regions. Numerous hepatocytes were undergoing degeneration or necrosis, with cellular fragmentation and karyolysis. Central veins were intact. By 40 mins, in centrilobular and midzonal regions, hepatocyte disassociation, degeneration, and necrosis was more marked with moderate hepatocyte loss. There was moderate centrilobular and mid-zonal hemorrhage with occasional loss of central veins. These lesions progressed and, at 60 mins, there was massive hepatocyte disassociation, degeneration, and necrosis with severe hepatocyte loss and severe hemorrhage throughout the entire liver lobule except for areas approximately 6 to 7 cells wide, surrounding

portal regions. Periportal hepatocytes occasionally were disassociated, and showed mild degeneration.

From 3 to 9 hours, the lesions progressed to include all of the liver parenchyma except for a layer of periportal hepatocytes 1 to 3 cells wide. From 9 to 24 hours, there was a progressive reduction in the number of cells and cellular debris within the hepatic parenchyma. Large areas of hemorrhage were present from 12 to 24 hours.

In the kidney, occasional tubules within the cortex contained small amounts of homogeneous, basophilic material beginning at 60 minutes, and progressing until, 18 and 24 hours, at which time numerous cortical tubules contained moderate amounts of this material. At 9 and 12 hours, small amounts of eosinophilic, fibrillar material with fine basophilic stippling (mineralization) were present within scattered glomerular capillaries and; by 18 and 24 hours, numerous glomerular capillaries contained moderate amounts of this same material.

In the lung, beginning at 1 hour, scattered capillaries and arterioles contained eosinophilic granular debris or intact rounded cells with abundant eosinophilic cytoplasm and pyknotic nuclei. These appeared to be hepatocytes. From 3 to 9 hours, numerous capillaries and arterioles contained increasing numbers of these intact cells while from 9 to 12 hours, the number of cells was decreased, but large amounts of globular, eosinophilic material (presumably necrotic cellular debris) were present. At 18 and 24 hours, fewer intact cells were seen intravascularly and the amount of eosinophilic, globular material also decreased.

3. <u>Serum Chemistry Alterations</u>

Statistically significant increases from control values were seen in liver related enzymes and in parameters evaluating renal function.

Beginning at 40 minutes, there was a marked increase in ALT which continued until the end of the study (Figures 3 and 4). ALP was markedly elevated commencing at 6 to 9 hours (Figure 5). Total bilirubin was elevated at 6 hours and was markedly increased by 9 to 12 hours (Figure 6). The BUN and creatinine were moderately elevated beginning at 9 to 12 hours and increase until the end of the study (Figures 7 and 8). The blood glucose concentration markedly decreased over time and phosphorous, although altered in a manner such that significant differences were seen at individual time points, did not vary in a consistent pattern over the 24-hour time period.

4. Sequential morphologic alterations in mice dosed with 7820 microcystin

Mice which were killed, or died at or after 30 minutes, had livers which were dark red and markedly enlarged. Liver and kidney weights increased with time (Table 2). Histologic lesions were similar in all affected animals at the same time points. Mice killed at 15 minutes had no significant microscopic lesions. At 30 minutes postdosing, mild centrilobular disassociation (pavementation) of hepatocytes was seen, and was occasionally accompanied by subcapsular congestion. By 60 minutes, the hepatic lesion had progressed to severe centrilobular congestion with disassociation and rounding up of hepatocytes, and individual cell necrosis. Hepatocyte degeneration was characterized initially by swelling, followed by increased cytoplasmic eosinophilia and nuclear pyknosis. One-third to one-half of the lobules were affected. A few neutrophils and lymphocytes were noted in affected areas. Free hepatocytes were seen in several central veins. In the kidney, mild lesions were first observed 60 minutes postdosing and became more severe by 90 minutes. These were characterized by mild dilation of cortical tubules, and many contained eosinophilic granular to fibrillar material.

Similar material was occasionally noted in Bowman's space and in glomerular capillaries. No treatment related lesions were seen in the lungs.

DISCUSSION

Based on our studies and those of others, the liver is the major target organ of 7820 microcystin. The marked hepatocyte disassociation and massive necrosis which occurred in rats in our experiments is similar to that reported in mice and sheep. No difference was noted in the response of male rats as compared to females, with no effect being seen below 80 ug/kg and virtually 100% lethality seen above 160 ug/kg. There is little apparent gradation of hepatic response in either mice or rats. After single exposure, animals are either clinically affected and die with massive liver destruction and hemorrhage, or the animals are clinically unaffected and no gross or histologic abnormalities are seen. In both rats and mice following ip injection, histologic lesions are present centrilobularly in the liver as early as 20-30 minutes. At 60 min., the histologic lesions in rats are as severe as those seen in mice at 60 min. However, mice die at 60 to 90 min. postdosing, but rats survive for 18 to 32 hours. Even with very high doses of 7820 microcystin the survival time in rats is still 6 to 8 hours.

A correlation exists between the initial histologic lesions seen via light microscopy, and a marked increase in ALT, beginning 40 minutes postdosing, which progresses to the end of the study. The early appearance and subsequent magnitude of the elevation in this enzyme's activity in the serum are indicators of, not only the brief interval between dosing and detectable liver injury (30 to 40 minutes), but also of the massive hepatic necrosis which ultimately occurs. In addition, there are marked increases in the SAP and total bilirubin. Although these are often correlated more with biliary obstruction, in this case they are also related to the massive

necrosis which occurs. Although serum glucose concentrations are initially very high, they fall precipitously to abnormally low values over the 24 hr. course of the study. This marked decrease in serum glucose may be due to the inability of the damaged liver to: 1) convert glycogen to glucose or 2) to synthesize glucose from precursor molecules.

In the liver, endothelial damage is evident by the massive hemorrhage and loss of central veins and sinusoids. Whether the initial effect is to endothelial cells in the liver, or to hepatocytes, with subsequent endothelial damage is unclear at this time. However, in vitro work utilizing hepatocytes and endothelial cells in culture will attempt to clarify this question. This breakdown in endothelial integrity, in conjunction with massive hepatocyte disassociation, appears to allow intact hepatocytes and necrotic cellular debris into the venous circulation and subsequently into the pulmonary artery. In rats, necrotic debris and intact cells, which are apparently degenerate hepatocytes, are seen in the pulmonary vasculature as early as 1 hour postdosing. The number of intact cells in the pulmonary vessels increases to a maximum at 6 to 9 hours, then beginning at 12 hours, they start to be gradually replaced by necrotic cellular debris until at 24 hours, very few intact cells are seen. It must be noted, however, that severe hepatic lesions precede the finding of intact cells and significant amounts of necrotic cellular debris in the pulmonary vasculature. To date, we have not seen such intact cells in the pulmonary circulation of mice although this has been reported[7].

Renal lesions in mice and rats are suggestive of glomerular damage with protein leakage into cortical tubules. These lesions are more extensive in rats than mice. This may be due to the presence of necrotic cellular debris circulating in glomerular capillaries. In rats, the presence of large amounts of this fibrillar material within glomerular capillaries at 18 and 24 hours

coincided with increased amounts of the homogeneous basophilic material within the lumens of renal cortical tubules. Renal cortical tubular necrosis was evident in rats at very high concentrations of 7820 microcystin. This may be due to a direct effect of the toxin on the tubular epithelium or on local renal circulation or be secondary to the effects of abnormal metabolic products and physiologic alterations resulting from shock induced by the massive hepatic necrosis and hemorrhage.

The time at which renal lesions were seen was roughly correlated to an elevation of the BUN and creatinine. These increases could be due to direct renal damage, a decreased rate of glomerular filtration from shock, or obstruction of glomerular capillaries with debris from the liver.

While the exact mechanism of action of the toxin produced by Microcystis aeruginosa remains to be determined, death in affected animals is presumably due to massive hepatic destruction and hemorrhage resulting in shock and loss of the myriad of liver functions necessary for the maintenance of life.

Table 1. Average liver and kidney weights (as % body weight) of rats given intraperitoneal injections of 7820 microcystin.

Dose ug/kg	n	Liver $\bar{x} \pm$ S.D (%)	Kidney $\bar{x} \pm$ S.D (%)
0*	3	4.93 + 0.51 −	0.9 + 0 −
20*	3	6.23 + 0.93 −	1.07 + 0.25 −
40*	3	3.70 + 0.26 −	0.77 + 0.15 −
80	1	4.20 + 0 −	1.00 + 0 −
120	2	4.70 + 0 −	1.05 + 0.07 −
160	7	4.73 + 0.39 −	1.01 + 0.07 −
200	3	5.17 + 0.49 −	1.03 + 0.06 −
240	1	4.10 + 0 −	1.40 + 0 −
400	3	6.13 + 0.050 −	1.10 + 0.12 −
800	3	6.33 + 1.29 −	1.03 + 0.21 −
1200	3	6.70 + 0.44 −	1.20 + 0.10 −

*All animals at these doses survived and were killed by ether inhalation at 2 to 12 days following treatment.

Table 2. Sequential liver and kidney weights (as % body weight) of mice injected intraperitoneally with 100 ug/kg of 7820 microcystin.

Time after dosing (min.)	Liver (% BW)	Kidney (% BW)
Controls (saline)	5.37	0.76
15	5.37	0.75
30	5.63	0.75
60	9.01	0.82
90	10.48	0.97
Spontaneous death (>90)	9.69	1.00

Figure 1: Relative liver weights (as % body weight) in rats (n = 2 to 7) injected ip with 160 ug 7820 microcystin/kg

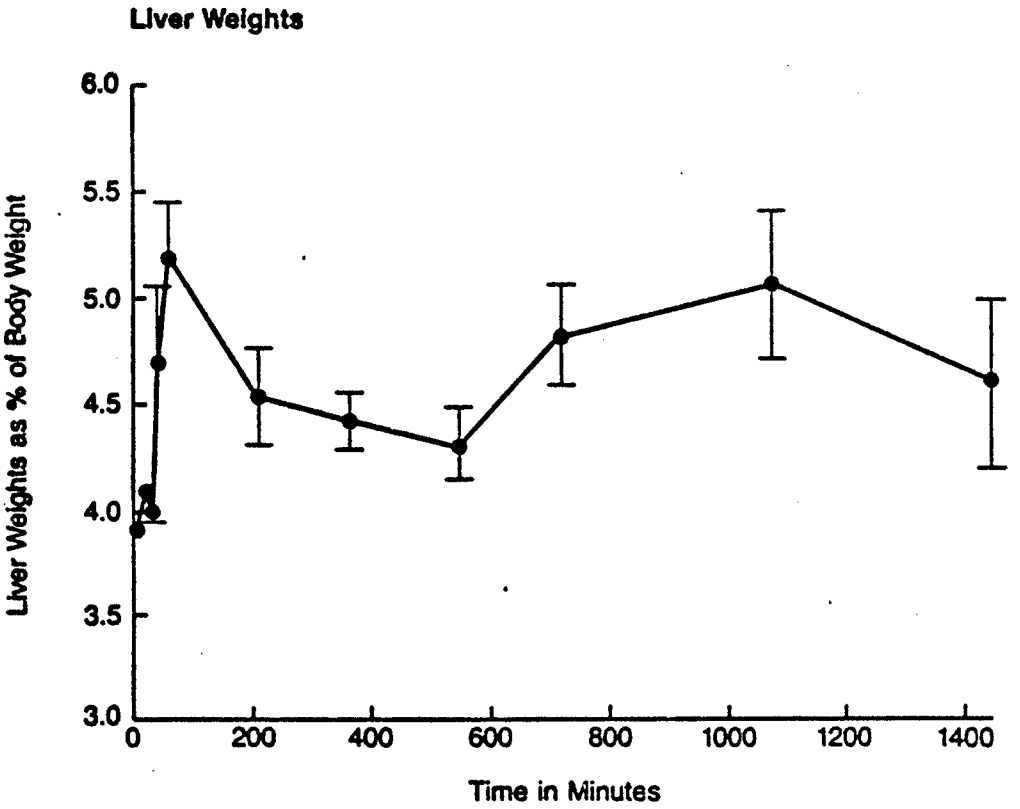


Figure 2: Relative kidney weights (as % body weight) in rats (n= 2 to 7) injected ip with 160 ug 7820 microcystin/kg

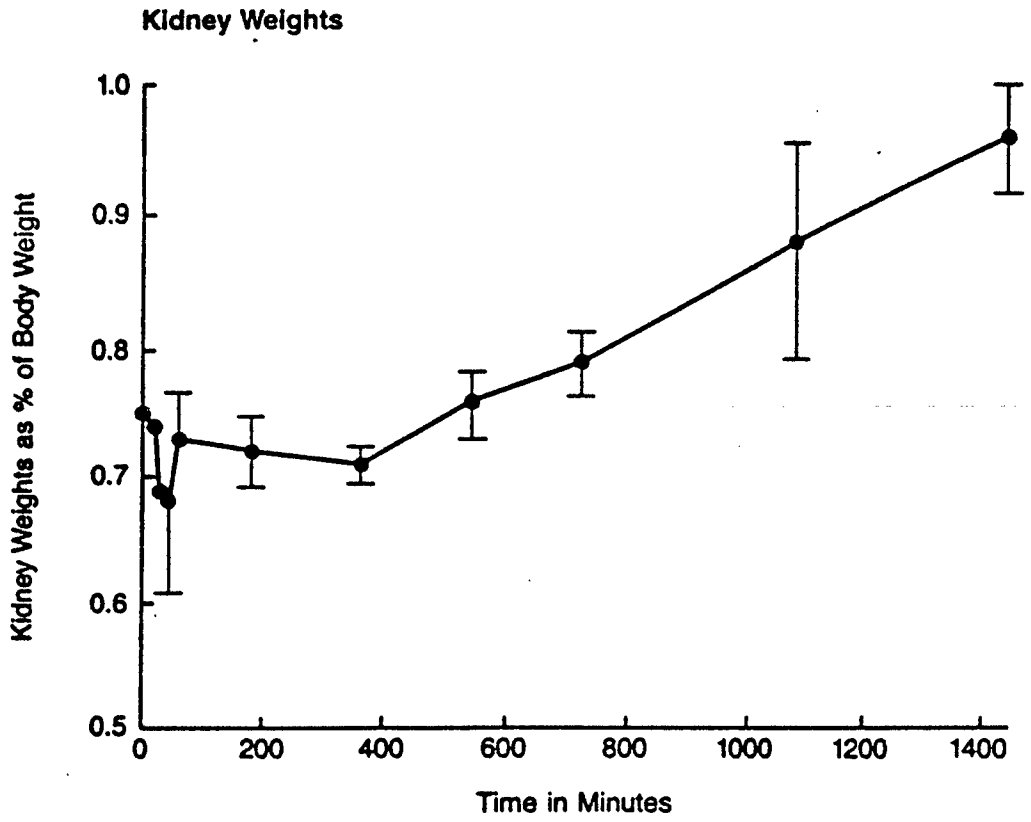


Figure 3: Alanine amino transferase (ALT, SGPT) in rats (n = 2 to 7) for the first 60 min. after ip injection with 160 ug/kg 7820 microcystin/kg

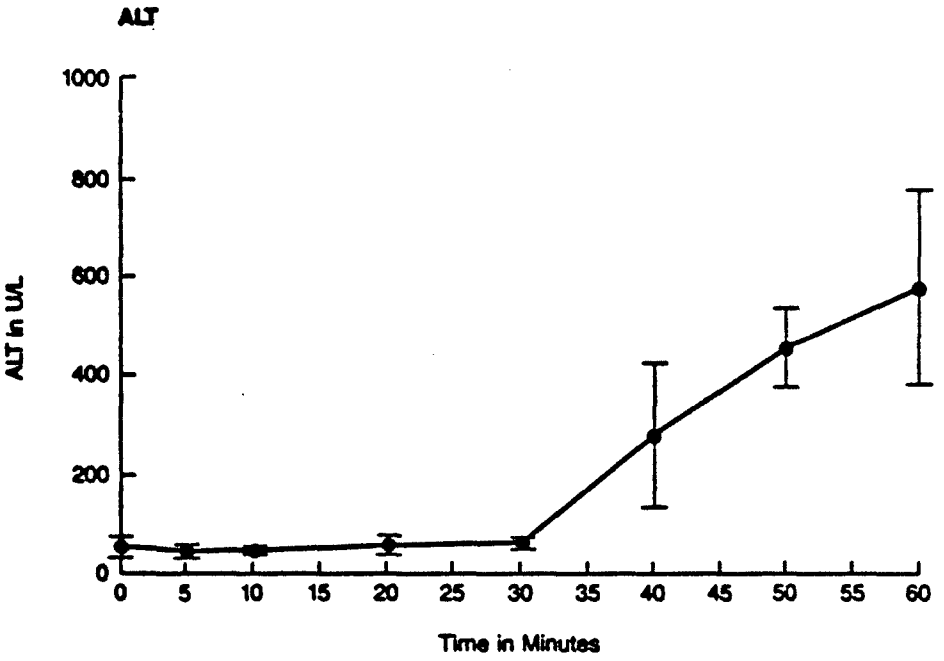


Figure 4: Alanine amino transferase (ALT, SGPT) in rats (n = 2 to 7) over the entire 24 hrs. period after ip injection with 160 ug/kg 7820 microcystin/kg

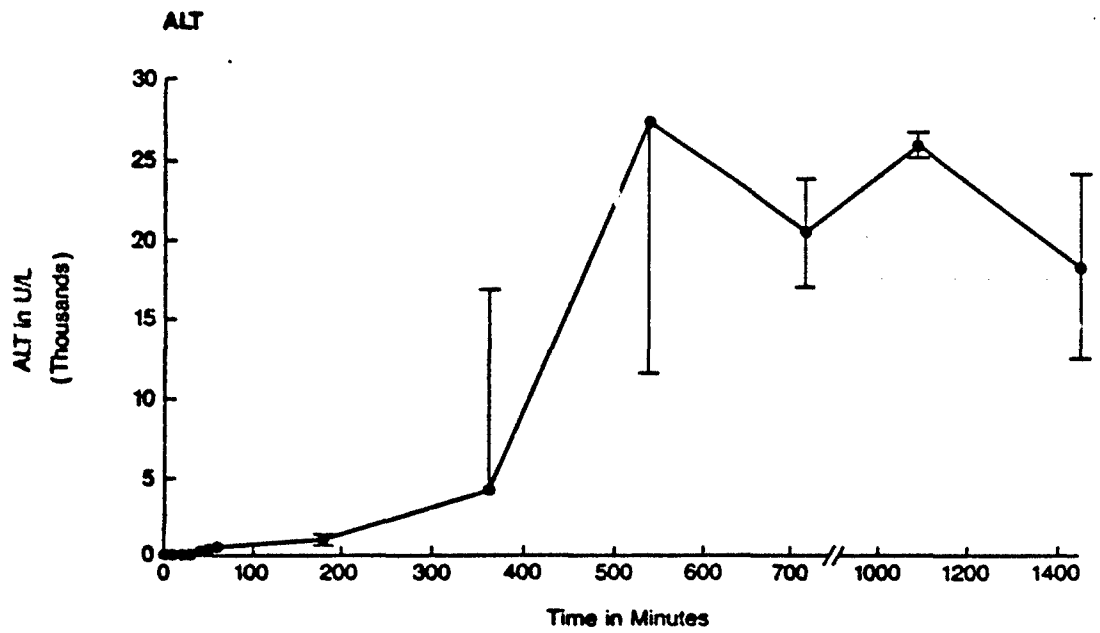


Figure 5: Serum alkaline phosphatase in rats (n = 2 to 7) after ip injection with 160 ug/kg 7820 microcystin/kg

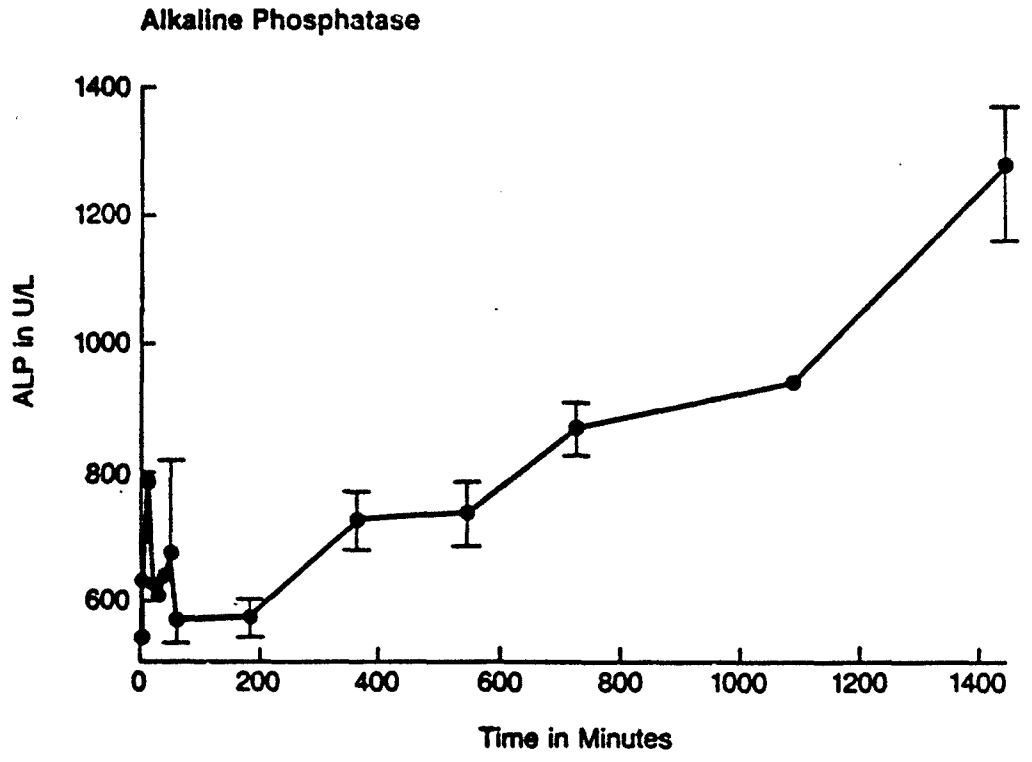


Figure 6: Total bilirubin in rats (n = 2 to 7) after ip injection with 160 ug/kg 7820 microcystin/kg

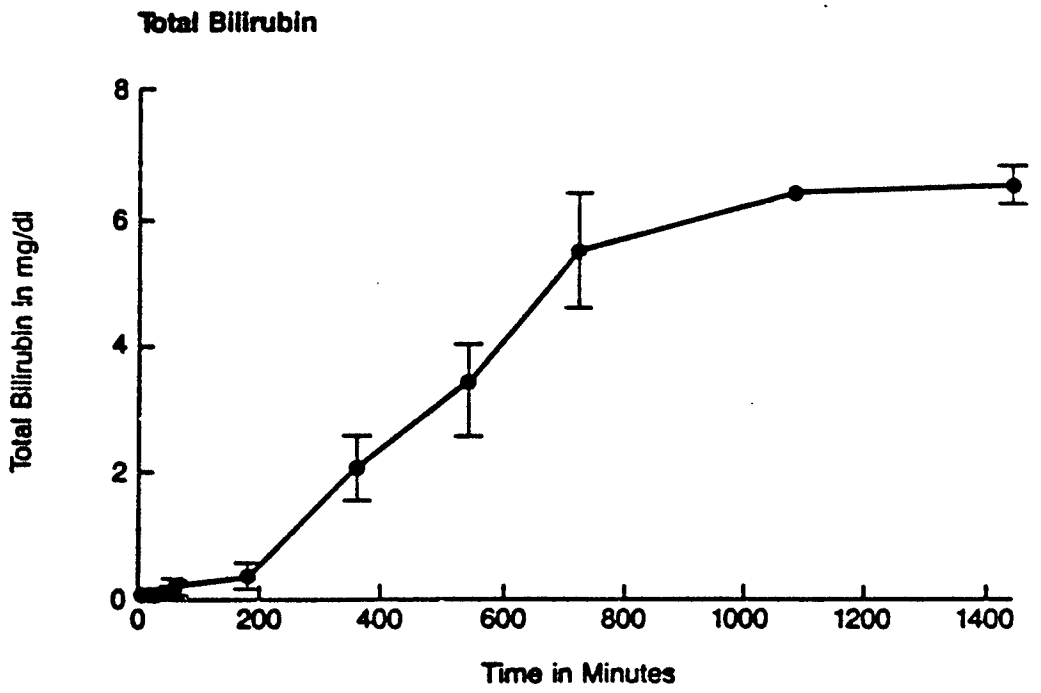


Figure 7: Blood urea nitrogen (BUN) in rats (n = 2 to 7) injected ip with 160 ug 7820 microcystin/kg

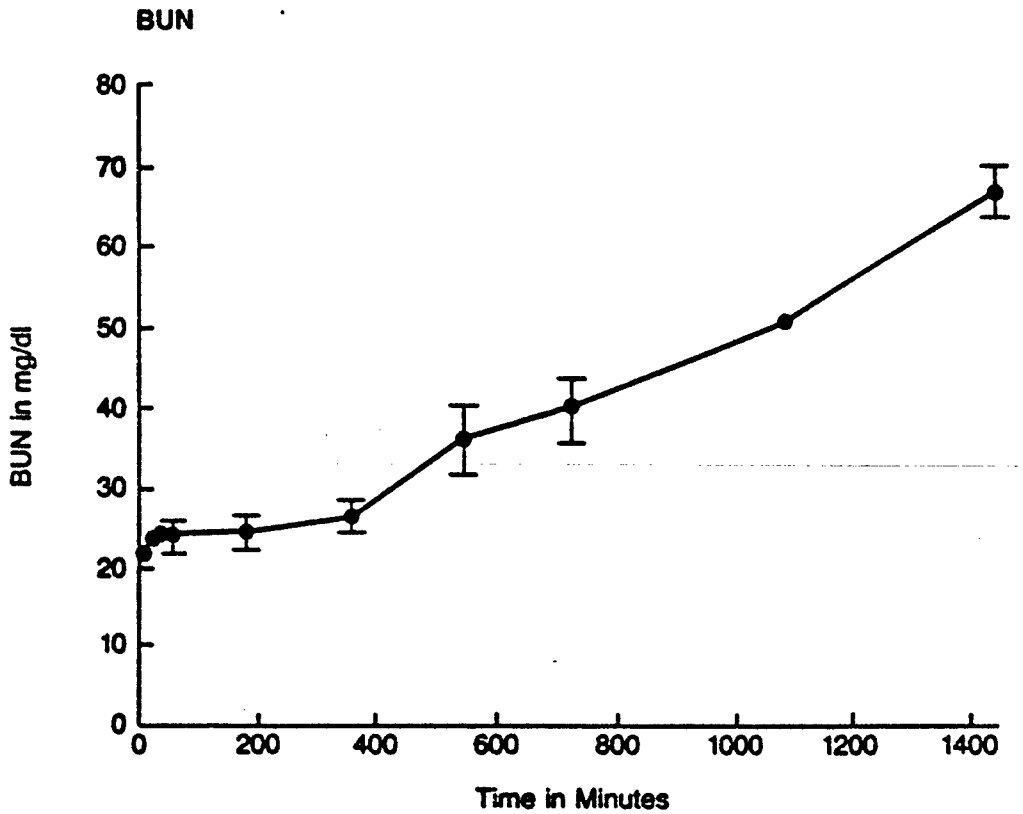
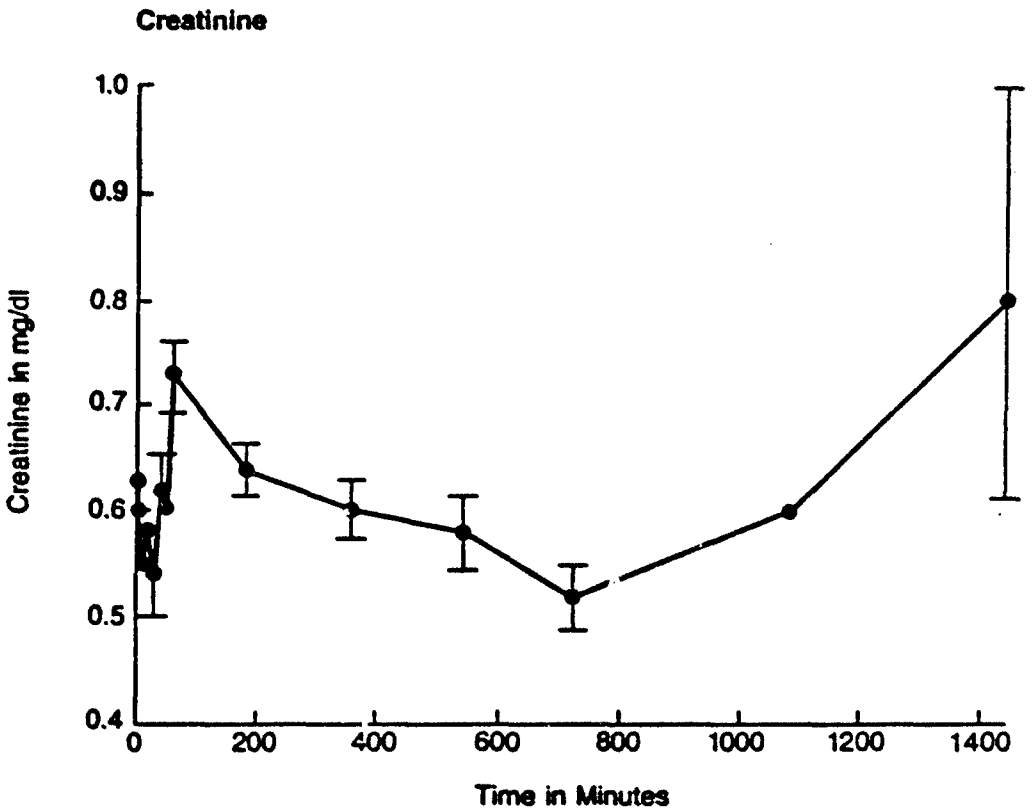


Figure 8: Serum creatinine in rats (n = 2 to 7) injected ip with 160 ug 7820 microcystin/kg



REFERENCES

1. Elleman, T., Falconer, I., Jackson, A., Runnegar, M. Isolation, characterization and pathology of the toxin from a Microcystis aeruginosa (=Anacystis cyanea) bloom. Aust. J. Biol. Sci. 1978. 31:1072-1076.

2. Falconer, I., Jackson, A., Langley, J., Runnegar, M. Liver pathology in mice in poisoning by the blue-green alga Microcystis aeruginosa. Aust. J. Biol. Sci. 1981. 34:179-187.

3. Falconer, I., Beresford, A.,, Runnegar, M. Evidence of liver damage by toxin from a bloom of the blue-green alga, Microcystis aeruginosa. Med. J. Aust. 1983. 1:511-514.

4. Jackson, A., McInnes, A., Falconer, I., Runnegar, M. Toxicity for sheep of the blue-green alga, Microcystis aeruginosa. Toxicon. 1983. suppl. 3:191-194.

5. Jackson, A., McInnes, A., Falconer, I., Runnegar, M. Clinical and pathological changes in sheep experimentally poisoned by the blue-green alga, Microcystis aeruginosa. Vet. Pathol. 1984. 21:102-113.

6. Galey, F., Beasley, V., Carmichael, W., Kleppe, G., Hooser, S., Haschek, W. Blue-green algae (Microcystis aeruginosa) hepatotoxicosis in a herd of dairy cows. In press.

7. Slatkin, D., Stoner, R., Adams, W. Atypical pulmonary thrombosis caused by a toxic cyanobacterial peptide. Science. 1983. 220:1383-1385.

8. Zin, L., Edwards, W. Toxicity of blue-green algae in livestock. Bov. Practitioner. 1979. 14:151-153.

DISTRIBUTION LIST

5 copies	Commander US Army Medical Research Institute of Infectious Diseases Attn: SGRD-UIZ-M Fort Detrick Frederick, MD 21701-5011
1 copy	Commander US Army Medical Research and Development Command Attn: SGRD-RMI-S Fort Detrick Frederick, MD 21701-5012
12 copies	Defense Technical Information Center (DTIC) Attn: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army Attn: AHS-CDM Fort Sam Houston, TX 78234-6100

END

DATE

FILMED

5-88

DTIC